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# Enzymatic Regulation of Opioid Antinociception and Tolerance

Lynn Hull

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# **Enzymatic Regulation of Opioid Antinociception and Tolerance**

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

By

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B.S. Worcester Polytechnic Institute 2002

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## List of Abbreviations

AC	Adenylyl Cyclase
ADPR	adenine dinucleotide phosphate ribose
ATP	Adenosine triphosphate
βARK	Beta Adrenergic Receptor Kinase
βNAD <sup>+</sup>	Beta Nicotinamide Adenine Dinucleotide
BST-1	Bone Marrow Stromal Cell Antigen 1
cADPR	Cyclic Adenine Dinucleotide Phosphate Ribose
cAMP	Cyclic Adenosine Monophosphate
CD157	Cell Surface Molecule 157 (also BST-1)
CD38	Cell Surface Molecule 38
cGDPR	Cyclic Guanine Dinucleotide Phosphate Ribose
CICR	Calcium Induced Calcium Release
Cx43	Connexin 43
DAG	Diacylglycerol
DAMGO	([D-Ala <sup>2</sup> , N-MePhe <sup>4</sup> , Gly-ol]-enkephalin) – high-efficacy opioid
DOR	δ-Opioid Receptor
ER	Endoplasmic Reticulum
GPCR	G-protein Coupled Receptor
GRK	G-protein Coupled Receptor Kinase
icv	Intracerebroventricular Injections
IP <sub>3</sub>	Inositol Triphosphate

KOR	κ-Opioid Receptor
MOR	μ-Opioid Receptor
NAADP <sup>+</sup>	Nicotinic acid adenine dinucleotide phosphate
NAD <sup>+</sup>	Nicotinamide Adenine Dinucleotide
NADP <sup>+</sup>	Nicotinamide Adenine Dinucleotide phosphate
NGD <sup>+</sup>	Nicotinamide Guanine Dinucleotide
NMN <sup>+</sup>	Nicotinamide Mononucleotide
NOP/FQ (N/OFQ)	Nociceptin/orphanin Receptor
PAG	Periaqueductal Grey
PKA	Protein Kinase A (also cAMP-dependent protein kinase)
PKC	Protein Kinase C
PLC	Phospholipase C
PM	Partition Mechanism
RyR	Ryanodine Receptor
sc	Subcutaneous Injection
Ser	Serine
SIM	Sequential Intermediate Mechanism
Thr	Threonine
VOC	Voltage-operated Ca <sup>2+</sup>
5'-AMP	5' Adenosine Monophosphate
7-deaza NHD <sup>+</sup>	Nicotinamide-7-deaza-hypoxanthine dinucleotide

## **Abstract**

### ENZYMATIC REGULATION OF OPIOID ANTINOCICEPTION AND TOLERANCE

By Lynn C. Hull, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of  
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Department of Pharmacology and Toxicology

The involvement of kinases in opioid actions has long been established. The acute actions of opioids, through the Gi/Go G-proteins, cause the inhibition of adenylyl cyclase and therefore a decrease in protein kinase A (PKA) activation. Additionally, acute opioid administration may cause the G-protein to activate the phospholipase C (PLC)-mediated cascade leading to the activation of protein kinase C (PKC).

The phosphorylation of the MOR which can lead to both desensitization by uncoupling of the G-protein coupled receptors (GPCRs) from the G-proteins and to internalization by recruitment of  $\beta$ -arrestins has long been identified as a key process in tolerance. Phosphorylation by PKA and PKC leads primarily to uncoupling of the receptor from the G-proteins. Phosphorylation of the receptor by G-protein coupled receptor kinase (GRK) leads to the recruitment of  $\beta$ -arrestins and internalization of the receptor. Many *in vitro* studies have come to the conclusion that GRK induced internalization plays a more

central role in the tolerance to high efficacy opioids and a lesser role in low- and moderate-efficacy opioid tolerance. In fact it has been hypothesized that morphine, a moderate-efficacy opioid, causes no internalization at all, while the desensitization of the receptor via phosphorylation by PKA and PKC plays a more central role in low- and moderate-efficacy opioid tolerance. We sought to test these *in vitro* findings in an *in vivo* model of opioid tolerance. Animals were made tolerant to one of a number of opioids of varying efficacy (low-efficacy meperidine, moderate-efficacy morphine and fentanyl, and high-efficacy [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO)) over an 8 hour period and then were administered one of the kinases' inhibitors. Tolerance reversal was determined by challenging these mice with the same opioids to which they were tolerant.

Calcium is known to play an important role in the acute antinociceptive actions of opioids as well as in opioid tolerance. Therefore it is important to determine how opioids are affecting the regulation of intracellular calcium. Our laboratory has previously shown that Calcium Induced Calcium Release (CICR), the ryanodine receptor and intracellular microsomal Ca<sup>2+</sup> pools all play a role in opioids' actions. It is also well known that mammalian ADP-ribosyl cyclase, CD38's, product cADPR acts on the ryanodine receptor to cause Ca<sup>2+</sup> release into the intracellular space. We chemically and genetically altered CD38 and then tested the acute effect of morphine as well as what effect these treatments had on morphine tolerance to determine what role if any, that CD38 may play in the acute actions of morphine antinociception as well as in morphine tolerance.

Together, studies focusing on the role of an ADP-ribosyl cyclase, CD38, and 3 separate kinases, PKA, PKC and GRK, in opioids' actions were performed in order to better understand the roles of these enzymes' pathways in the actions of opioid-induced antinociception and subsequent development of tolerance. It is hoped that the results herein add useful knowledge to the general understanding of this drug class, and will one day be of use in the development of future analgesics and in the clinical treatment of pain and reduction in tolerance.



# CHAPTER 1

## Background

### I. Opioid History

Opioids have been used for their analgesic properties for thousands of years with the earliest recorded use found in the writings of Theophrastus in the third century B.C. Today, in addition to their use as potent analgesics, they are used clinically for their potent antitussive and antidiarrheal qualities.

Opioids are a class of compounds which are related to the opiate morphine. The term opiate refers to drugs which are directly derived from the *Papaver somniferum* poppy plant, while the term opioid refers to all drugs in this class whether they are from natural or synthetic origin. Opioids' effects are mediated by the same system of receptors and pathways as endogenous opioids (also called endogenous opioid peptides or endorphins) of which there are over a dozen. There are four major receptor types including the mu-opioid receptor  $\mu$  (MOR), kappa-opioid receptor  $\kappa$  (KOR), delta-opioid receptor  $\delta$  (DOR), and the N/OFQ receptor.

Morphine was first isolated in 1806 as a distinct alkaloid from opium by Sertürner. This discovery was quickly followed by the isolation of other alkaloids from opium and led to the synthesis of new opioid compounds. The drive for synthesis of new opioids was in large part based on the search for an opioid which retained its analgesic properties but did not possess the common side effects and addictive potential that classical opioids share.

However, all of the drugs in this class of compounds have the same side effects, although the degree varies from chemical to chemical, and all share abuse liability.

The endogenous opioid system was first suggested when a unique receptor was proposed from the observation by Beckett and Casy in 1954 that opiate ligands seem to share unique structural requirements (Beckett *et al.*, 1954a; Beckett *et al.*, 1954b). In the 1970s there were a number of advances in definitively describing an opioid system which is responsible for the effects of opioids. Liebeskind's group was able to show that direct electrical stimulation of the periaqueductal grey (PAG) area of the brain in rats results in analgesia which could be blocked with the administration of the opioid antagonist naloxone (Akil *et al.*, 1976; Akil *et al.*, 1972). Early in 1973, evidence of specific receptors for opioids was found in experiments where binding of the radio labeled opioid antagonist naloxone was competed with by a number of other opioid agonists and antagonists (Pert *et al.*, 1973). Additionally similar experiments using radio labeled etorphine showed the same competitive binding results in rat brain (Simon *et al.*, 1973) with the receptor being located in the plasma membrane fraction (Terenius, 1973). From these early reports of an opioid receptor it was not long before the discovery of the first endogenous opioid, enkephalin, took place in 1975 by the Kosterlitz laboratory. Two enkephalins have been identified, methionine-enkephalin and leucine-enkephalin (Hughes *et al.*, 1975). The fact that opioid receptors are stereo specific was determined by Goldstein's lab in 1976 (Goldstein, 1976). Also in 1976, the Martin lab postulated multiple opioid receptors. They were the mu-opioid receptor (MOR) named after the ligand morphine, the kappa-opioid receptor (KOR) named after the ligand

ketocyclazocine and the sigma-opioid receptor (later found not to be an opioid receptor) named for the ligand SKF-10,047 (Martin *et al.*, 1976). They also characterized the different effects of these agonists and respective opioid receptor activation in dogs. It is also now known that there is the delta-opioid receptor (DOR). A fourth receptor in the opioid family was cloned in 1994 by three separate labs independently (Bunzow *et al.*, 1994; Fukuda *et al.*, 1994; Mollereau *et al.*, 1994). All three labs were looking for a receptor with similar structural characteristics shared by the other three receptors. The receptor was identified as the nociceptin/orphanin FQ (N/OFQ) receptor (NOP). Further discussion of the N/OFQ receptor is not relevant to these studies and is beyond the scope of this introduction.

The MOR, KOR and DOR are often called the classical opioid receptors and share several characteristics. Structurally, they are all G-protein coupled receptors (GPCRs) and have the characteristic seven transmembrane regions typical of GPCRs with homology in the membrane spanning and cytoplasmic domains. In the transmembrane domain they share the ability of the third intracellular loop to bind with the Gi G-protein to inhibit adenylyl cyclase (Kurose *et al.*, 1983), with the Go G-protein to inhibit voltage gated  $\text{Ca}^{2+}$  channels (Hescheler *et al.*, 1987), and with both Gi and Go G-proteins activating the inwardly rectifying  $\text{K}^{+}$  channels in the plasma membrane (Hescheler *et al.*, 1987). Opioids' actions through the MOR cause an increase in  $\text{K}^{+}$  conductance and a decrease in  $\text{Ca}^{2+}$  conductance which together cause the membrane to have reduced excitability (Chavkin *et al.*, 1988; Childers, 1993; North, 1993; North *et al.*, 1987). The resulting hyperpolarization of the membrane potential may be the cause of the blockade

of neurotransmitter release and pain transmission caused by opioids (North, 1993; Williams *et al.*, 1988; Wimpey *et al.*, 1991).

Agonists such as etorphine and levorphanol and antagonists such as naloxone, naltrexone and diprenorphine bind to opioid receptors. However, these receptors are very different as well. Typically MOR and DOR have more characteristics in common with each other than either has with KOR receptors. MOR and DOR are able to undergo agonist-mediated internalization through an endocytic pathway while KOR does not internalize after prolonged agonist exposure (Chu *et al.*, 1997; Gaudriault *et al.*, 1997; Trapaidze *et al.*, 1996). MOR agonists are the most commonly used clinically of the three receptor agonists. They produce analgesia, affect mood and behavior, and alter the respiratory, cardiovascular, gastrointestinal and neuroendocrine systems (Pasternak, 1988; Wood, 1998). DOR agonists are most commonly used as analgesics in animals, and only occasionally in humans (Coombs *et al.*, 1985) while KOR agonists can be used as analgesics although they are rarely used due to the dysphoric and psychomimetic effects they cause (Pfeiffer *et al.*, 1986).

All three opioid receptor types are found throughout the body. They are widely expressed in the periphery including tissues such as the small and large intestine, adrenal glands, kidney, lung, spleen, testies, ovaries and uterus (Wittert *et al.*, 1996). In the central nervous system the opioid receptors are also widespread. The analgesic effects of opioids are mediated by the MOR, DOR and KOR at both supraspinal and spinal levels. The MOR shows higher selectivity over the DOR and KOR supraspinally in the PAG, median

raphe nuclei, magnus raphe nuclei, gigante cellular reticulum and spinally in both the spinal cord and the dorsal root ganglia (Mansour *et al.*, 1995).

Chronic exposure to opioids leads to the development of tolerance. This is a multifaceted response which leads to a reduced effectiveness of the opioid after repeated administration. Although there has been extensive research done to determine the exact mechanisms of tolerance, there is still much to be learned. Tolerance is known to involve at least three different types of mechanisms each of which may be reliant on a number of pathways. These mechanisms are desensitization, internalization and sequestration/degradation of the receptor.

Desensitization can be defined as a decrease in the cellular response to further agonist stimulation upon continuous or repeated exposure to the agonist (Benovic *et al.*, 1988). The desensitization in the opioid receptor system most likely involves phosphorylation of the receptor by a number of different kinases including protein kinase C (PKC) (Mestek *et al.*, 1995; Narita *et al.*, 1995), protein kinase A (PKA) and G-protein coupled receptor kinase (GRK) (Pei *et al.*, 1995; Wang *et al.*, 1994). There are two types of desensitization; heterologous desensitization and homologous desensitization. Heterologous desensitization is the cross-regulation of receptors where activation of one receptor can cause desensitization of another. Additionally, in this type of desensitization the stimulation of the receptor by one agonist can lead to a broad pattern of unresponsiveness to further stimulation by a variety of other agonists. Homologous desensitization is the desensitization of only the agonist bound active receptors and is directly responsible for

the activation of the desensitization response. Although the difference in which type of desensitization is employed in different opioids' tolerance is an important concept, it is beyond the scope of this dissertation.

The internalization of the receptor occurs through a classical endocytic pathway; however the pathways are slightly different for MOR and DOR which suggests that the mechanisms may be receptor specific and involve different intracellular trafficking mediators (Gaudriault *et al.*, 1997). The internalization process is also ligand specific, where some ligands cause rapid internalization of the MOR receptor (etorphine and DAMGO for example) while others (such as morphine) are less able or cannot cause MOR internalization, although they are still able to cause other tolerance mechanisms such as desensitization (Keith *et al.*, 1998; Keith *et al.*, 1996; Whistler *et al.*, 1998). These differences may be due to altered conformational changes to the receptor which are dependent on the ligands and can cause divergent intracellular events to occur. Opioid receptor internalization is caused in part by the phosphorylation of the receptor by GRK. GRK causes only homologous phosphorylation in that it selectively acts on the receptor which is activated. The phosphorylation of these receptors promotes the interaction with  $\beta$ -arrestins which both interfere with G-protein coupling and cause receptor internalization (Bohn *et al.*, 1999).

## **II. Protein kinase A, Protein kinase C, and G-protein coupled receptor kinases**

### **IIa. PKA**

Adenylyl cyclase (AC) is activated by the active G-protein subunit  $G_{\alpha}$ . Adenylyl cyclase is an integral protein in the plasma membrane with its active site on the cytosolic side of the membrane. When AC is activated by the G-protein it causes the synthesis of cAMP from ATP and raises the intracellular concentration of cAMP. cAMP is degraded to 5'-AMP by cyclic nucleotide phosphodiesterase.

Protein Kinase A (aka cAMP-dependent protein kinase) is allosterically activated by cAMP. The inactive form of PKA has 4 subunits, two are catalytic subunits and two are regulatory subunits. The tetrameric PKA complex is inactive because the association of the regulatory subunits (R) with the catalytic subunits (C) blocks the catalytic domains on the catalytic subunits with autoinhibitory domains on the regulatory subunits. When four cAMP molecules bind to two sites on each regulatory domain there is a conformational change and the tetrameric complex dissociates revealing two free catalytically active C subunits. These active C subunits are then able to phosphorylate serine (Ser) and threonine (Thr) residues that are surrounded with the specific sequence which PKA is required to bind.

The active site of PKA is in a 240 residue section of the catalytic subunit. This section is highly conserved in all kinases and it is responsible for the binding of the substrates, both ATP and the target sequence surrounding the Ser or Thr residue to be phosphorylated. The specificity of PKA is due to several glutamic acid residues which form salt bridges

with two arginine residues in the target sequence of the bound peptide. These types of interactions both stabilize the reaction and make the interactions between the different kinases and their targets specific.

There are specific isoforms of PKA. These isoforms are created through varying the catalytic and regulatory subunits which make up PKA. Each subunit is encoded by a specific gene and there are four known regulatory subunits and two known catalytic subunits (Brandon *et al.*, 1997; Cadd *et al.*, 1989). Although none of the isoforms of PKA have yet to be implicated in opioid tolerance, all of the regulatory and catalytic subunits are expressed in neurons.

### Iib. PKC

Phospholipase C (PLC) is an enzyme in the plasma membrane which is activated by Gq and catalyses the formation of two second messengers diacylglycerol (DAG) and inositol 1,3,4-triphosphate (IP<sub>3</sub>) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate, a lipid in the plasma membrane. PLC hydrolyzes the bond between the glycerol and phosphate in phosphatidylinositol 4,5-bisphosphate releasing IP<sub>3</sub>, which is water soluble, and DAG, which remains associated with the plasma membrane. IP<sub>3</sub> acts on the IP<sub>3</sub> receptors on the ER to cause release of Ca<sup>2+</sup> into the cytosol. This cytosolic Ca<sup>2+</sup> increase, in conjunction with the membrane bound DAG, causes the activation of PKC. PKC phosphorylates Ser and Thr residues on specific target proteins with specific sequences surrounding the residues to be phosphorylated. PKC catalyzes the transfer of a phospho group from ATP to a specific residue on the target protein sequence.



There are a number of different isoforms of PKC. Work from Zeitz, Hua and our laboratory have shown that the PKC $\alpha$ ,  $\gamma$ , and  $\varepsilon$  but not PKC  $\beta$ I,  $\beta$ II,  $\delta$ ,  $\theta$ ,  $\varepsilon$ ,  $\eta$ , or  $\xi$  isoforms appear to contribute to the expression of morphine tolerance in mice (Hua *et al.*, 2002; Smith *et al.*, 2007; Zeitz *et al.*, 2001). It is interesting to note that all of the PKC isoforms which have been found to play a role in morphine tolerance are also all dependent on calcium.

### IIc. GRK

Originally identified as  $\beta$ -adrenergic receptor kinase ( $\beta$ ARK), this kinase is a member of the G protein coupled receptor kinases (GRK) family. These kinases phosphorylate Ser residues near the carboxyl terminus of receptors. The cytosolic kinase is recruited to the plasma membrane where it performs its phosphorylation by association with the Gs $\beta\gamma$  subunit. The phosphorylation of the receptor by GRK allows for the binding of  $\beta$ -arrestins which disrupt function of the receptor and target it for internalization through endocytosis. Once internalized in small intracellular vesicles, the receptors are dephosphorylated and either recycled to the plasma membrane to be active receptors again or are targeted for degradation. There are at least 5 different GRKs and 4 different arrestins which enables this family of kinases to act on a number of different receptors in a regulated manner.

## **III. Opioids and CD38**

It has been well documented that the stimulation of receptors coupled to the Gi/Go G-protein, such as the MOR, is associated (through the activation of K<sup>+</sup> channels and the

resultant hyperpolarization of the membrane) with the inhibition of the voltage-operated  $\text{Ca}^{2+}$  (VOC) channels in the plasma membrane, and therefore with the prevention of the elevation of intracellular free calcium (Hille, 1994). However, there is also evidence that opioid stimulation can also cause a transient increase in intracellular  $\text{Ca}^{2+}$  (Connor *et al.*, 1996; Tomura *et al.*, 1992; Yeo *et al.*, 2001). Many enzymes which regulate intracellular calcium have been investigated for their potential role in opioids' actions; however the  $\text{Ca}^{2+}$  regulating enzyme CD38 has yet to be examined in this way. Therefore, we chose to determine if CD38 plays a role in opioids' actions as well.

#### **IV. CD38**

CD38 is a type II transmembrane glycoprotein which is a part of a family of enzymes with ADP ribosyl cyclase activity. This family of enzymes is able to cyclize  $\text{NAD}^+$  into cADPR. Additionally they are able to perform other enzymatic functions as well. CD38 itself is an ectoenzyme which is secured on membranes, both plasma and intercellular, and performs its cyclization function on the non-cytosolic side of the membrane.

##### IVa. ADP-ribosyl cyclase Family

CD38 is a member of the ADP-ribosyl cyclase superfamily of enzymes. It is the prototypic mammalian member of the family in addition to CD157 (also known as BST-1) (Hirata *et al.*, 1994). The original member of this family, found in 1991 in the invertebrate *Aplysia californica*, is the *Aplysia* ADP-ribosyl cyclase which is a soluble protein (Lee *et al.*, 1991). All three of these ADP-ribosyl cyclase proteins share a common gene organization and enzymatic reaction mechanisms indicating that they share

a common ancestor (Cakir-Kiefer *et al.*, 2000; Dong *et al.*, 1996; Nata *et al.*, 1997; Yamamoto-Katayama *et al.*, 2002). The family shares phylogenetic origins, structural conformation and biological functions (Ferrero *et al.*, 1999; Katz *et al.*, 1983), and both of the genes which encode for CD157 and CD38 can be found on chromosome 4. There are some differences in the enzymatic functions of the three members of this family. Namely the ratios of cADPR to ADP-ribose (ADPR) produced. *Aplysia* ADP-ribosyl cyclase produces mainly cADPR and very little ADPR, while the mammalian cyclases CD38 and CD157 produce a majority of ADPR and only a small amount of cADPR (Schuber *et al.*, 2004).

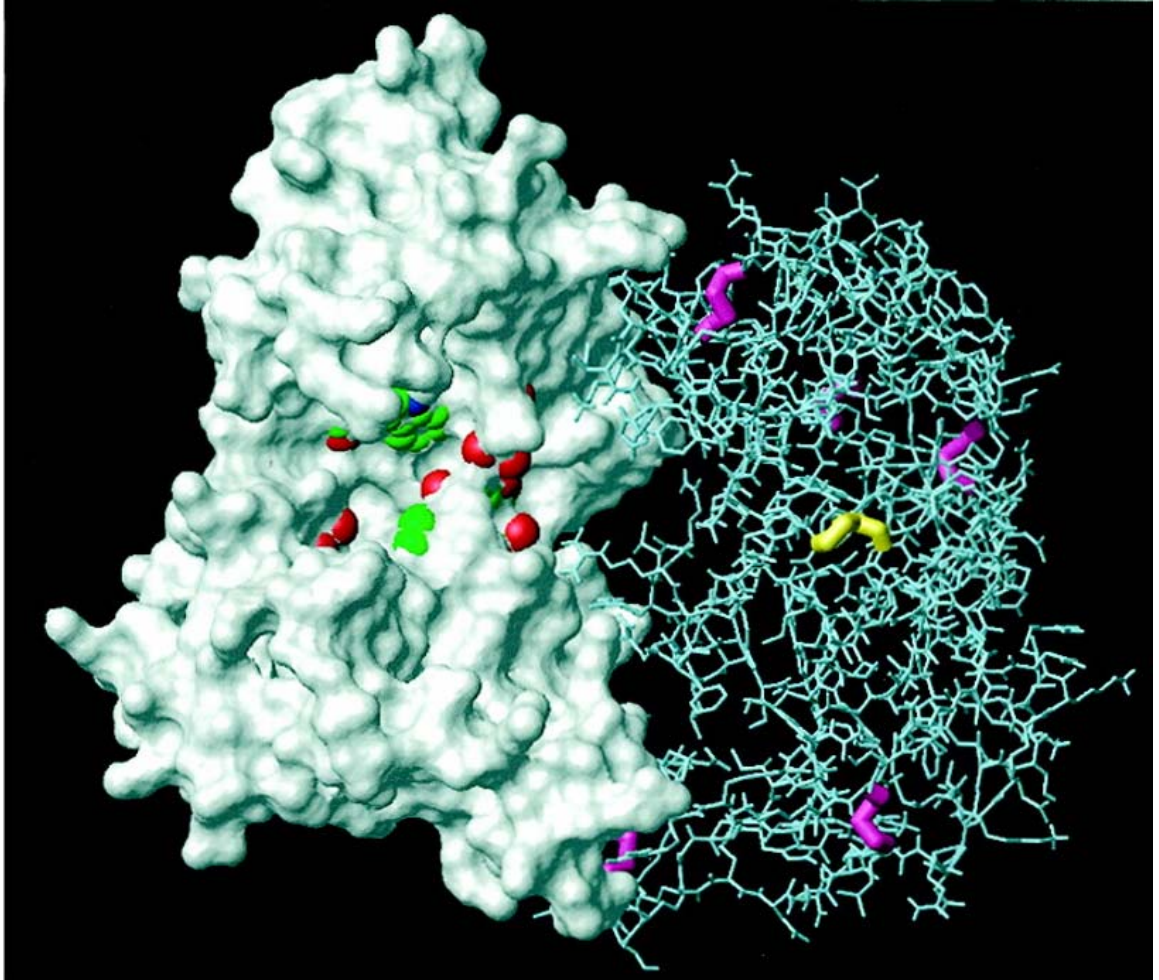
#### IVb. CD38 Structure

CD38 as a monomer is a 45 kDa protein which is a type II glycoprotein, and so its carboxy terminus is found outside the membrane, with a short cytoplasmic tail, a transmembrane region and an extracellular domain (Jackson *et al.*, 1990; Malavasi *et al.*, 1992). Prasad's model of CD38's structure, based on homology with the *Aplysia* cyclase, indicates a disulfide bridge in a hinge region connecting the major domains of two monomers in the formation of a dimer (Prasad *et al.*, 1996). The action of the disulphide bridge is important for catalytic activity (Tohgo *et al.*, 1994) and may play a role in the activity related conformational changes in CD38 (Prasad *et al.*, 1996). Hinge motion during the catalytic activity of CD38 can be expected and may play a role in the so called topological paradox of CD38, allowing its extracellular product to reach intracellular space (Franco *et al.*, 1998). CD38 is able to form homodimers and oligomers which have a higher ADP-ribosyl cyclase activity than the monomer. These multimers are stable and

are the result of disulphide bridges similar to the insulin receptor cross linking in the membrane via disulphide bridges into a larger functionally active form (Chidambaram *et al.*, 1998). Chidambaram found that the eluted proteins of monomer, dimer and oligomers of CD38 converted  $\text{NGD}^+$  into cGDPR at the rate of 34.55, 60.79, and 215.07 nmols/mg protein/minute, respectively. It has not been determined if there is any increase in the CD38 channel activity with the homotetramers versus homodimers as there has been in catalytic activity, but the monomeric form seems to only have catalytic activity and no influx capacity (Franco *et al.*, 1998).

The CD38 protein is made up of three regions; a 20 amino acid intracellular region, a 23 amino acid transmembrane region and a 257 amino acid extracellular region which includes the binding site for the oligosaccharide residues which make this a glycoprotein (Alessio *et al.*, 1990). The crystal structure of *Aplysia* ADP-ribosyl cyclase may give some insight as to why there are multimer forms of CD38. The structure suggests that the enzyme contains two spatially separated pockets which are made up of sequences of conserved residues. Prasad *et al.* suggest that the transmembrane homodimeric structure would generate a central channel in the protein structure (Prasad *et al.*, 1996). If that hypothesis is correct then the dimerized form of CD38 may be forming a cavity with these pockets in the extracellular space where  $\text{NAD}^+$  can bind to be converted to cADPR. The pocket region may then allow for a hinge motion to occur leading to the transport of cADPR into the cell (Deaglio *et al.*, 2001; Prasad *et al.*, 1996).

**Figure 1: Homology model of the catalytic domain of CD38**



The model is based on the crystallography coordinates of a dimer of *Aplysia* ADP-ribosyl cyclase. Two identical monomers of CD38 are shown with one rendered with van der Waals contact surface and the other is represented as wire frame. The five conserved disulfide bonds are depicted as purple cylinders, and the non-conserved one is colored yellow. (Munshi *et al.*, 2000)

#### IVc. Function

CD38 was originally of interest as a marker of immune cell activation and is studied in HIV infection, leukemias, myelomas, solid tumors, type II diabetes mellitus and bone metabolism. It can be used as a prognostic marker in leukemia and to identify plasma cells (Deaglio *et al.*, 2001; Hasmoni *et al.*, 2009; Rawstron, 2006) and may also function as a receptor on hematopoietic cells with functions on the immune cells including cell adhesion, signal transduction and calcium signaling (Kumagai *et al.*, 1995; Lund *et al.*, 1999).

The first evidence that CD38 was more than a surface antigen and in fact had an enzymatic role in mammalian cells was reported first in a murin model and confirmed in humans in 1993 (Gelman *et al.*, 1993; Howard *et al.*, 1993). CD38 functions enzymatically as an ADP-ribosyl cyclase, a cyclic ADP-ribose hydrolase and a NAD<sup>+</sup> glycohydrolase (Zocchi *et al.*, 1993). The classical mammalian NAD<sup>+</sup> glycohydrolase has now been identified as CD38 (Augustin *et al.*, 2000; Berthelieir *et al.*, 1998). These catalytic functions allow the enzyme to synthesize cADPr, ADPR and NAADP, all of which have distinct calcium mobilizing activities (Lee, 2001).

CD38 was originally defined and studied in the immune system as a T lymphocyte differentiation antigen (Reinherz *et al.*, 1980), but has since been found to be expressed throughout the body in many different cell and tissue types including the brain (Higashida *et al.*, 1997; Lee *et al.*, 1993; Malavasi *et al.*, 1992; Partida-Sanchez *et al.*, 2001; Stashenko *et al.*, 1981; Tedder *et al.*, 1984; Walseth *et al.*, 1997). Mizuguchi found

in 1995 using immunochemical and immunohistochemical means that CD38 is expressed in the adult human brain. They were able to localize CD38 to the perikarya (cell body) and dendrites of many neurons indicating a potential signal transduction role in the neurons of the central nervous system (Mizuguchi *et al.*, 1995).

One of CD38's products, cADPr, has also been implicated in playing a role in many neuronal activities such as synaptic transmission, neurotransmitter release and calcium events of rhythmic bursting (Budde *et al.*, 2000; Reyes-Harde *et al.*, 1999; Verderio *et al.*, 2001). This potential role in signal transduction in the CNS fits with CD38's role in mobilizing intracellular  $\text{Ca}^{2+}$  through the generation of its second messenger cADPR. It is known that the alteration in intracellular  $\text{Ca}^{2+}$  in neurons controls many of the neuron's functions including plasticity (Berridge, 1993a).

#### IVd. cADPR and Calcium

One of CD38's products, cADPr, was first discovered as a calcium mobilizing metabolite of NAD in 1987 (Clapper *et al.*, 1987) and its structure was resolved in 1994 (Kim *et al.*, 1993b; Lee *et al.*, 1994; Lee *et al.*, 1989). Since then, cADPR has been found to mobilize calcium in many different organisms and cell types (Cancela, 2001; da Silva *et al.*, 2000; De Flora *et al.*, 2000; Galione *et al.*, 2000; Guse, 2000; Guse, 2002; Guse, 1999; Higashida *et al.*, 2001; Lee, 2000a; Lee, 2000b; Lee, 2001; Okamoto *et al.*, 2000).

In neurons the homeostatic mechanisms that tightly control cytosolic free  $\text{Ca}^{2+}$  levels are important in regulating many functions, such as membrane excitability and second

messenger pathways that involve adenylyl cyclase and the phosphatidylinositol system (Choi *et al.*, 1992; Im *et al.*, 1992). The ability of neurons to release  $\text{Ca}^{2+}$  from intracellular pools plays a major role in regulating levels of cytosolic  $\text{Ca}^{2+}$ . The ryanodine receptor (RyR) pool releases  $\text{Ca}^{2+}$  on stimulation by either cADPR or by  $\text{Ca}^{2+}$  and caffeine (Ashley, 1989; McPherson *et al.*, 1990; Teggatz *et al.*, 2005; Zhang *et al.*, 2006). Within neurons, this pool is involved with the phenomenon of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) in which  $\text{Ca}^{2+}$  entry into the cytosol, through 1,4-dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels, amplifies its signal by stimulating RyR to release pooled  $\text{Ca}^{2+}$  (Friel *et al.*, 1992). Additionally  $\text{Ca}^{2+}$  released from these pools acts as a positive feedback to trigger further release by activating subsequent RyRs (Berridge, 1993b).

In neurons and neuron related cell types, cADPr has been mainly reported to act on the ryanodine receptor on the endoplasmic reticulum (Galione, 1993; Higashida *et al.*, 2000; Hua *et al.*, 1994; Meszaros *et al.*, 1993). The RyR, named for its identification with the exogenous vegetal alkaloid ryanodine, is located in the brain and peripheral tissues classically on the endoplasmic reticulum (De Flora *et al.*, 1997). In neurons, low concentrations of the plant alkaloid ryanodine slightly stimulate  $\text{Ca}^{2+}$  release, whereas 1 $\mu\text{M}$  ryanodine blocks  $\text{Ca}^{2+}$  release and accumulation by either preventing channel opening or by stabilizing an open subconductance state (McPherson *et al.*, 1991; Rousseau *et al.*, 1987).

In brain tissues, RyR is found to be primarily type 3 which, in addition to RyR type 2, is stimulated by cADPR at low calcium concentrations (Guse, 1999; Hakamata *et al.*, 1994;



Meszaros *et al.*, 1993; Sonnleitner *et al.*, 1998). The stimulation of the ryanodine receptor by cADPR causes  $\text{Ca}^{2+}$  to be released into the cytosolic space from the stores in the endoplasmic reticulum (Currie *et al.*, 1992). The ryanodine receptor is one of two known intracellular calcium release channels, the other being the inositol 1,4,5 triphosphate receptor ( $\text{IP}_3$ ), both of which are found in many types of central neurons (Ferris *et al.*, 1992; Kuwajima *et al.*, 1992; McPherson *et al.*, 1991; Supattapone *et al.*, 1988). The ryanodine receptor is also sensitive to caffeine and calcium (Bezprozvanny *et al.*, 1991; McPherson *et al.*, 1991) and has been identified as a key part of the calcium induced calcium release (CICR) mechanism in neurons (Kuba, 1980; Kuwajima *et al.*, 1992; Lipscombe *et al.*, 1988; Marengo *et al.*, 1996; Marrion *et al.*, 1992; Reyes *et al.*, 1996; Shmigol *et al.*, 1995; Simpson *et al.*, 1996). CICR, mediated by the ryanodine receptor may be an important mechanism mediating many neuronal functions which depend on increases in the intracellular calcium concentration (Berridge, 1993a; Hua *et al.*, 1994). cADPR was found to act on the ryanodine receptors in experiments by Currie *et al.* where the ryanodine pool of calcium was dumped by stimulating the receptor with calcium and kept from refilling by maintaining the cells in a calcium deficient environment (Currie *et al.*, 1992). The cells were then exposed to cADPR and there was no further  $\text{Ca}^{2+}$  release found in these cells indicating that the pool of calcium that is acted on by cADPR is the same as that regulated by ryanodine (Currie *et al.*, 1992). Additionally there have been reports of caffeine and calcium sensitizing the effects of cADPR as well as antagonists of the ryanodine receptor such as ruthenium red, procaine, and high concentrations of ryanodine and  $\text{Mg}^{2+}$  inhibiting the calcium release caused by cADPR (Lee, 1997). cADPR has been found to modulate the RyR calcium stores in both neurons and

astrocytes (Budde *et al.*, 2000; Empson *et al.*, 1997; Hashii *et al.*, 2000; Hua *et al.*, 1994; Verderio *et al.*, 2001), and has been implicated in many calcium-dependent neural processes such as synaptic transmission, plasticity and neuronal excitability (Brailoiu *et al.*, 2000; Budde *et al.*, 2000; Mothet *et al.*, 1998; Reyes-Harde *et al.*, 1999; Verderio *et al.*, 2001).

#### IVe. Topological Paradox

CD38 is an ectoenzyme and so its enzymatic activity is located on the extracellular side of the membrane. This presents a distinct problem as its products are intracellularly acting calcium mobilizers. This paradox, often referred to as a topological paradox, is two fold; the substrate for the enzyme,  $\text{NAD}^+$ , is found primarily intracellularly, and the product of interest, cADPR, is produced extracellularly but acts intracellularly at the RyR on the endoplasmic reticulum.

The answer to the first part of this paradox is to transport the substrate  $\text{NAD}^+$  out of the cell so that CD38 has access to it. There are a few ways in which the pyridine dinucleotide,  $\text{NAD}^+$ , can pass out of the cell. In particular in many mammalian cell types a passive, temperature independent  $\text{NAD}^+$  transport system has been found on the plasma membrane (Zocchi *et al.*, 1999). This transmembrane  $\text{NAD}^+$  transporter is known to be connexin 43 (Cx43) (Bruzzone *et al.*, 2001). Connexons are hexameric hemichannels which exist either in homomeric or heteromeric forms containing one or a number of different connexin isoforms. They have been found to be membrane-spanning structures which can be lined up in two adjacent cells to form gap junction channels (Bruzzone *et*

*al.*, 1996; Kumar *et al.*, 1996). These types of channels have long been known to exchange many small molecules including second-messengers and small metabolites between cells and between the intracellular and extracellular space of a single cell (Bruzzone *et al.*, 1996; Rhee *et al.*, 1996).

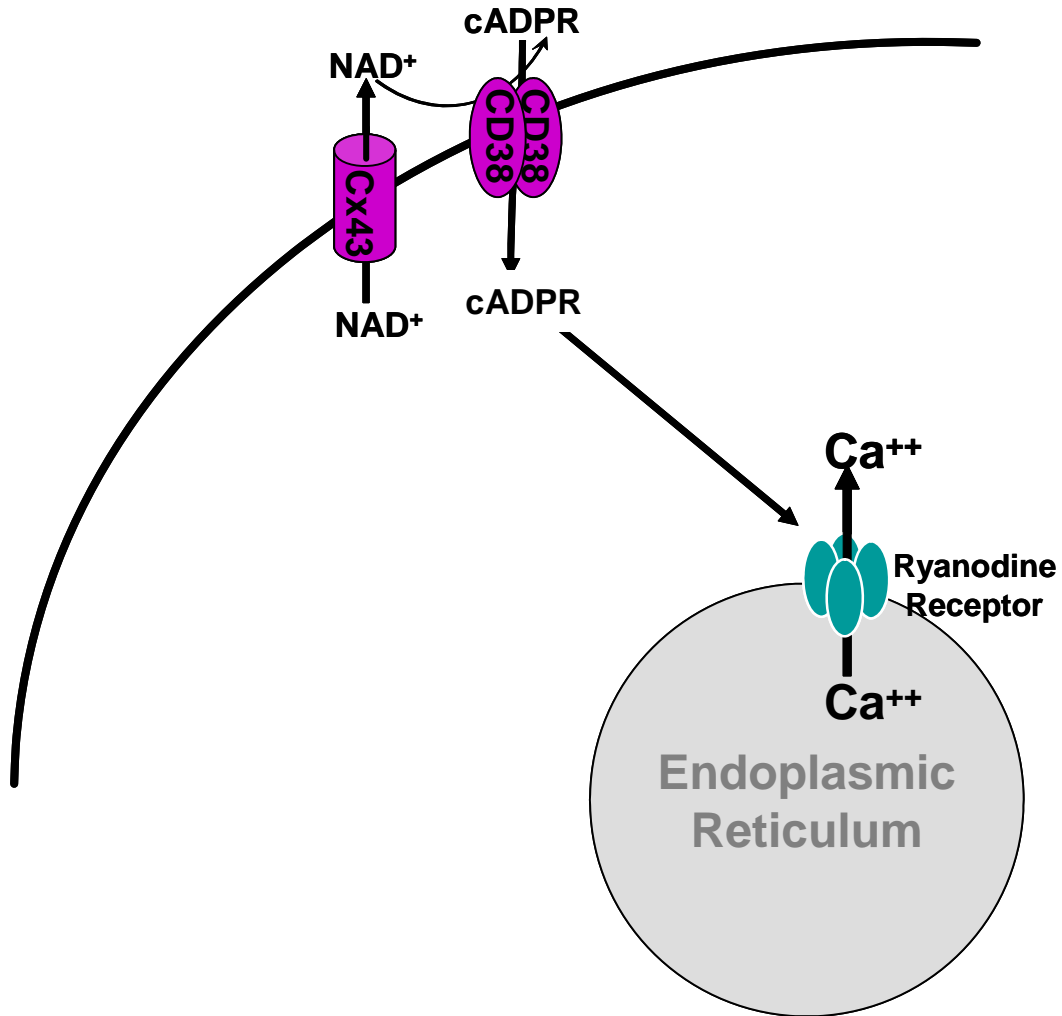
Cx43 has not only been identified as a transporter of  $\text{NAD}^+$ , but it has been found that a buildup of intracellular  $\text{Ca}^{2+}$  can function as an inhibitor of the channel thus prohibiting unnecessary loss of  $\text{NAD}^+$  from the cell and potentially dangerous levels of  $\text{Ca}^{2+}$  from accumulating in the cell (Bruzzone *et al.*, 1996; Simon, 1999; Unger *et al.*, 1997; Zocchi *et al.*, 1999). The Cx43 channel also has shown specificity in transport inhibition experiments (Bruzzone *et al.*, 2001). In these experiments there was no transport of either cADPR or ADPR both of which are metabolically related nucleotides to  $\text{NAD}^+$ . This is in agreement with other findings in the literature that the isoform composition of connexin channels determines their selective permeabilities to second-messenger and small metabolite molecules (Bevans *et al.*, 1998). This same characteristic selectivity of connexin channels suggests that there may be another connexin channel which selectively transports cADPR across the membrane (De Flora *et al.*, 1996; Podesta *et al.*, 2000). Such a channel has been suggested to occur in lens cells (Churchill *et al.*, 1998).

In addition to the potential connexin transport of cADPR into the cell, as suggested to happen in lens cells, there are other means for solving the second part of the paradox of how to get cADPR into the cell so that it can reach its destination at the RyR. Of particular interest is the ability of oligomeric CD38 to couple their production of cADPR

with an active translocation of the molecule across the plasma membrane to the intercellular space (Bruzzone *et al.*, 1998; Franco *et al.*, 1998; Moreno-Garcia *et al.*, 2004). This transport is saturation dependent and occurs against a concentration gradient (Franco *et al.*, 1998). It has been reported that the 3-dimensional model of CD38 has a solvent accessible cavity (Prasad *et al.*, 1996). If two (or more) CD38 monomers join to form a dimer (or oligomer) a channel can be formed in the center. This proposed channel may be how CD38 is able to transport cADPR into the cell (De Flora *et al.*, 1997) with a hinge action taking place after the formation of cADPR has occurred. Additionally this transport is only available to the cADPR made in the CD38 oligomer. Studies where excess cADPR was added externally resulted in the failure of the excess cADPR to cross the membrane at levels that would be expected if the CD38 oligomers were transporting any extracellular cADPR besides that which the enzyme made (Franco *et al.*, 1998).

It is also possible that due to the localization of CD38 and the specific properties of its catalytic site that a significant fraction of cADPR produced might escape transport across the membrane by CD38 (Franco *et al.*, 2001). The fact that cADPR is not membrane permeant suggests that there may be additional means for its transport across the cell membrane. There have been such CD38-unrelated transport systems for cADPR reported in native, constitutively CD38<sup>-</sup>, 3T3 fibroblasts (Franco *et al.*, 2001). Both equilibrative and concentrative nucleoside transporters have been found to be responsible for the cADPR translocation, and these transporters have been found in a wide variety of mammalian cells (Baldwin *et al.*, 1999; Cass, 1999; Ritzel *et al.*, 2001; Wang *et al.*, 1997).

**Figure 2: CD38's "Topological Paradox" solved**



CD38's substrate,  $\text{NAD}^+$ , is transported out of the cell by channels such as Cx43 where it is converted into cADPR which is then transported back into the cell by the channel formed by the CD38 dimer. Once in the cell it is able to stimulate the ryanodine receptor on the endoplasmic reticulum to cause release of calcium into the cytosolic space.

The variable and multifaceted transport of  $\text{NAD}^+$  and cADPR across the cell membrane indicates that the topological paradox is not actually a paradox at all, but perhaps a unique means by which the alteration of intracellular  $\text{Ca}^{2+}$  by cADPR can be regulated through the regulation of the transport of the substrate and the product to and from the enzyme.

## **V. Rational for using the PAG**

The PAG was chosen as the main brain area for the *ex vivo* studies because it is known to play an important role in modulating the activity of bulbospinal monoaminergic antinociceptive systems (Aston-Jones *et al.*, 1991; Reichling *et al.*, 1988). The PAG has a high density of MOR and is the location of the  $\beta$ -endorphin containing terminals of cell bodies from the arcuate nucleus. There is also evidence that the PAG plays an important role in opioids' actions as well as in the interplay between  $\text{Ca}^{2+}$  and opioids. It has been found that injections of  $\mu$ -opioids directly into the PAG results in dose-dependent antinociception as well as a decrease in the levels of interneuronal  $\text{Ca}^{2+}$ , and that repeated injections of opioids into the PAG results in tolerance (Akaike *et al.*, 1978; Budai *et al.*, 1998; Jacquet *et al.*, 1974; Jacquet *et al.*, 1976; Lewis *et al.*, 1977; Mayer *et al.*, 1971; Reynolds, 1969; Sandkuhler *et al.*, 1991; Siuciak *et al.*, 1987; Tortorici *et al.*, 1999; Yaksh *et al.*, 1976; Zhang, 1992). Experiments have shown that opioids inhibit GABAergic neurons in the PAG and that this inhibition of GABAergic transmission produces antinociception by disinhibiting PAG output neurons (Basbaum *et al.*, 1984; Behbehani *et al.*, 1990a; Behbehani *et al.*, 1990b; Reichling *et al.*, 1990; Vaughan *et al.*, 1997a; Vaughan *et al.*, 1997b). It is in our favor that the PAG coincidentally lines the

aqueduct between the third and fourth ventricle and because of this placement is exposed to any drugs which are injected icv into the lateral ventricles of the brain.

## **VI. Limitations of Intracerebroventricular Injection**

Because the PAG is readily accessible to the kinase inhibitors, CD38 modulators, and opioids injected intracerebroventricularly (icv) in these studies, it is tempting to speculate that this region may be solely responsible for morphine antinociception and tolerance. However, there are limitations to this model. It is known that the expression of morphine tolerance also involves the spinal cord and peripheral sites, therefore alterations of opioid actions only in the PAG may affect opioids' actions and tolerance only in that brain region while leaving other important regions responsible for antinociception and tolerance unaffected.

It is likely that such a complex phenomenon such as analgesia may include other areas of the body as well, and future research should be done to study these potential interactions, as well as the relative importance these regions play. It is just as likely that one area is dominant over the others as it is that all of the areas play an equal role but at separate time points in the generation of morphine induced antinociception, and these interactions need to be elucidated as well.

## **VII. Calcium and Opioids**

There have been reports of an increase in intracellular  $\text{Ca}^{2+}$  after opioid receptor activation alone, but more commonly it has been reported to happen during concomitant activation of Gq-coupled receptors which cause the  $\text{Ca}^{2+}$  release from intracellular stores

(Connor *et al.*, 1996; Okajima *et al.*, 1993). Regardless of whether the increase of  $\text{Ca}^{2+}$  results from MOR activation alone, or with another class of GPCR, it is clear that the majority of this elevation of intracellular  $\text{Ca}^{2+}$  is a result of the release of  $\text{Ca}^{2+}$  from stores in the ER rather than through an influx of  $\text{Ca}^{2+}$  across the plasma membrane (Werry *et al.*, 2003) (Jin *et al.*, 1992; Okajima *et al.*, 1993). Opioids have been reported to stimulate the inositol phosphate turnover in many cell types (Chueh *et al.*, 1995; Dortch-Carnes *et al.*, 2003; Smart *et al.*, 1996; Zimprich *et al.*, 1995) which is correlated with the release on  $\text{Ca}^{2+}$  from the  $\text{IP}_3$  mediated stores. However, there is also evidence that there may be other mechanisms by which the opioids increase the level of intracellular  $\text{Ca}^{2+}$  such as through activation of the ryanodine receptors (Allouche *et al.*, 1996). It has been shown that the ryanodine receptor antagonist dantrolene blocks morphine induced elevation in intracellular  $\text{Ca}^{2+}$  in isolated mouse astrocytes (El-Hage *et al.*, 2005; Hauser *et al.*, 1996).

Opioid receptor activation and resulting stimulation of the Gi/Go G-proteins usually results in inhibition of neurotransmitter release (Christie *et al.*, 2000). However the brief elevations in pre-synaptic  $\text{Ca}^{2+}$  levels after opioid receptor stimulation can be enough to stimulate neurotransmitter release as well (Cahill *et al.*, 1993; McDonald *et al.*, 1996; Xu *et al.*, 1992). There have also been reports that, in addition to the opioid receptors being coupled to the inwardly rectifying  $\text{K}^+$  channels to cause membrane hyperpolarization and depression of neuronal excitability, the opioid receptor may be linked to  $\text{Ca}^{2+}$ -sensitive  $\text{K}^+$  channels such as the BK(Ca) channel (Chin *et al.*, 2002; Ramnarine *et al.*, 1998; Shirasaki *et al.*, 2001). If this is the case then the increase in  $\text{Ca}^{2+}$  caused by opioid



stimulation could further enhance postsynaptic inhibition by opening these  $\text{Ca}^{2+}$ -sensitive  $\text{K}^{+}$  channels.

The ability of opioids to cause elevations in intracellular  $\text{Ca}^{2+}$  implies that  $\text{Ca}^{2+}$  signaling molecules may be important in mediating opioid induced antinociception (Samways *et al.*, 2006) (Aoki *et al.*, 2003; Narita *et al.*, 2000; Xie *et al.*, 1999). There is much experimental evidence for the role of  $\text{Ca}^{2+}$  in acute opioid antinociception. Agents which increase cytosolic  $\text{Ca}^{2+}$  in intact cultured neurons and synaptosomes isolated from the CNS are able to block opioid antinociception when injected icv. Hano reported in 1964 that intracisternal administration of  $\text{Ca}^{2+}$  ions antagonize morphine antinociception (Hano *et al.*, 1964). Additionally, other methods of increasing the levels of  $\text{Ca}^{2+}$  also affect opioid antinociception. Ionophores X-537A and A23187, which increase intracellular  $\text{Ca}^{2+}$  (Pressman, 1976) were shown to block opioid antinociception (Harris *et al.*, 1975; Vocci *et al.*, 1980). These types of experiments led to the hypothesis that  $\text{Ca}^{2+}$  alters intracellular events to antagonize the antinociceptive effects of opioids (Chapman *et al.*, 1980). Additionally, experiments using  $\text{Ca}^{2+}$  chelators, such as EGTA and EDTA, as well as  $\text{Ca}^{2+}$  channel antagonists, such as verapamil, diltiazem and dihydropyridine, resulted in the potentiation of antinociception in animals (Ben-Sreti *et al.*, 1983; Benedek *et al.*, 1984; Carta *et al.*, 1990; Contreras *et al.*, 1988; Del Pozo *et al.*, 1990; Hoffmeister *et al.*, 1986; Horvath *et al.*, 1990; Seyler *et al.*, 1983). Similar results have been found in humans as well. In clinical research,  $\text{Ca}^{2+}$  channel antagonists have been found to enhance opioid analgesia in cancer patients and surgical patients without increasing respiratory depression (Boldt *et al.*, 1987; Carta *et al.*, 1990; Pereira *et al.*, 1993;

Santillan *et al.*, 1994) or increasing the rewarding properties of opioids (Vaupel *et al.*, 1993).

It is also known that a disruption in intracellular  $\text{Ca}^{2+}$  homeostasis contributes to the expression of antinociceptive tolerance. Opioid tolerance causes synaptosomal  $\text{Ca}^{2+}$  uptake to be increased (Chapman *et al.*, 1980) and basal free-intracellular  $\text{Ca}^{2+}$  concentrations are found to be higher in the brain and spinal cord compared to non-tolerant samples (Harris, 1976; Welch *et al.*, 1991; Yamamoto *et al.*, 1981). It has also been found that the density of the 1,4-dihydropyridine  $\text{Ca}^{2+}$  channel is increased in morphine tolerance in both rat and mouse brain (Ramkumar *et al.*, 1984; Ramkumar *et al.*, 1988). Opioid tolerance can also be affected by the alteration of the intercellular  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  channel antagonists have been shown to prevent the development of opioid tolerance (Dierssen *et al.*, 1991), reverse tolerance (Antkiewicz-Michaluk *et al.*, 1993; Contreras *et al.*, 1988; Dierssen *et al.*, 1991) and attenuate the signs of physical dependence in animals (Antkiewicz-Michaluk *et al.*, 1993; Baeyens *et al.*, 1987; Bongianini *et al.*, 1986; Ramkumar *et al.*, 1988; Silverstone *et al.*, 1992). Additionally, previous work in our lab has shown that extracellular  $\text{Ca}^{2+}$  influx through voltage-sensitive  $\text{Ca}^{2+}$  channels, and mobilization from  $\text{Ca}^{2+}$ /Caffeine-sensitive pools (RyR pools) are involved with morphine tolerance (Smith *et al.*, 1999) and can be reversed with the application of ryanodine. It has also been demonstrated that when  $\text{Ca}^{2+}$  is injected into the PAG, opioid antinociception from any type of injection, systemic or otherwise, is blocked (Munoz *et al.*, 1982). Previous work in our lab has shown that this block in antinociception by injecting  $\text{Ca}^{2+}$  is a result of the stimulation of  $\text{Ca}^{2+}$  influx into the cells

and the subsequent  $\text{Ca}^{2+}$  release from ryanodine receptor pools through calcium induced calcium release (CICR) (Smith *et al.*, 1995). In these experiments thapsigargin, a sesquiterpene lactone isolated from the roots of *Thapsia garganica* L, was administered. Thapsigargin is a selective inhibitor of the  $\text{ATP/Mg}^{2+}$ -dependent ATPase and so blocks the uptake of  $\text{Ca}^{2+}$  into  $\text{IP}_3$ -sensitive microsomal pools (Bian *et al.*, 1991; Koshiyama *et al.*, 1991). In neurons the administration of thapsigargin and subsequent depletion of the  $\text{IP}_3$   $\text{Ca}^{2+}$  pool leads to an increase in free intracellular  $\text{Ca}^{2+}$  (Foskett *et al.*, 1992; Jackson *et al.*, 1988). By injecting thapsigargin into the PAG our lab was able to increase intracellular levels of  $\text{Ca}^{2+}$  which in turn blocked morphine antinociception. We hypothesized that the increase in intracellular  $\text{Ca}^{2+}$  was the cause of the ability of thapsigargin to block morphine's antinociceptive effects, thus implicating fluctuations in intracellular  $\text{Ca}^{2+}$  levels as a key component in morphine antinociception and tolerance.

## **VIII. Scope of Dissertation**

### VIIIa. CD38, Calcium and Opioids

It is clear that  $\text{Ca}^{2+}$  plays an important role in the acute antinociceptive actions of opioids as well as in opioid tolerance. Therefore it is an important next step to determine how opioids are affecting the regulation of intracellular calcium. Our laboratory has previously shown that CICR, the ryanodine receptor and intracellular microsomal  $\text{Ca}^{2+}$  pools all play a role in opioid's actions. It is also well known that CD38's product cADPR acts on the ryanodine receptor to cause  $\text{Ca}^{2+}$  release into the intracellular space. We chemically and genetically altered CD38 and then tested the acute effect of morphine

as well as what effect these treatments had on morphine tolerance to determine what role if any, that CD38 may play in the acute actions of morphine antinociception as well as in morphine tolerance.

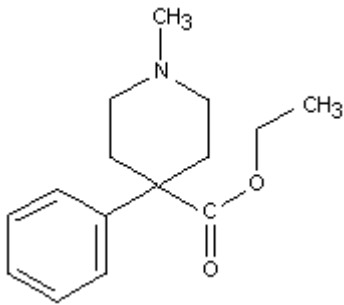
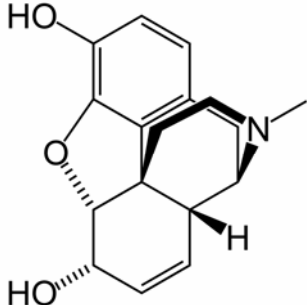
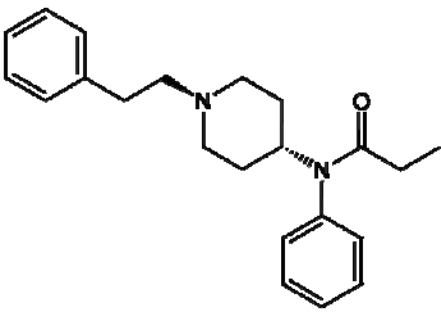
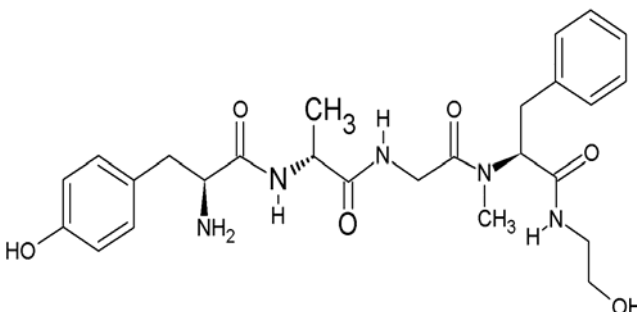
#### VIIIb. PKA, PKC, GRK and Various Efficacy Opioid Tolerance

The involvement of kinases in opioid actions has long been established. The acute actions of opioids, through the Gi/Go G-proteins, cause the inhibition of adenylyl cyclase and therefore a decrease in PKA activation (Herz, 1993; Sharma *et al.*, 1977). Additionally, acute opioid administration may cause the G-protein to activate the phospholipase C (PLC)-mediated cascade leading to the activation of PKC (Akil, 1988).

The phosphorylation of the MOR which can lead to both desensitization, by uncoupling of the GPCR from the G-proteins, and to internalization by recruitment of  $\beta$ -arrestins has long been identified as a key process in tolerance. Phosphorylation by PKA and PKC leads primarily to uncoupling of the receptor from the G-proteins. Phosphorylation of the receptor by GRK leads to the recruitment of  $\beta$ -arrestins and internalization of the receptor. Many *in vitro* studies have come to the conclusion that GRK induced internalization plays a more central role in the tolerance to high efficacy opioids and a lesser role in low- and moderate-efficacy opioid tolerance. In fact it has been hypothesized that morphine, a moderate-efficacy opioid, causes no internalization at all, while the desensitization of the receptor via phosphorylation by PKA and PKC plays a more central role in low- and moderate-efficacy opioid tolerance. We sought to test these *in vitro* findings in an *in vivo* model of opioid tolerance. Animals were made tolerant to one of a number of opioids of

varying efficacy (low-efficacy meperidine, moderate-efficacy morphine and fentanyl, and high-efficacy [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO)) over an 8 hour period and then were administered one of the kinases' inhibitors. Tolerance reversal was determined by challenging these mice with the same opioids to which they were tolerant.

**Table 1: Structure of opioids used**

 <p><b>Meperidine</b></p>	 <p><b>Morphine</b></p>
 <p><b>Fentanyl</b></p>	 <p><b>DAMGO</b></p>

The opioids used in these studies were low-efficacy meperidine, moderate-efficacy morphine, moderate-efficacy fentanyl and high-efficacy DAMGO ([D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin).

#### VIIIc. Overall Scope of this Dissertation

Together, studies focusing on the role of an ADP-ribosyl cyclase, CD38, and 3 separate kinases, PKA, PKC and GRK, in opioids' actions were performed in order to better understand the roles of these enzymes' pathways in the actions of opioid-induced antinociception and subsequent development of tolerance. It is hoped that the results herein add useful knowledge to the general understanding of this drug class, and will one day be of use in the development of future analgesics and in the clinical treatment of pain and reduction in tolerance.

## **CHAPTER 2**

### **The Effect of PKC, PKA and GRK Inhibition on Tolerance Induced by $\mu$ -opioid Agonists of Different Efficacy**

#### **I. Summary**

Differences in tolerance and receptor desensitization resulting from exposure to opioid agonists of different efficacy have been reported. The objective of this study was to determine the effects of PKC, PKA and GRK inhibition on antinociceptive tolerance *in vivo* to opioid agonists of different efficacy. An 8 hour tolerance model was used where an opioid was repeatedly administered to naïve mice. Animals were challenged with the opioid following injection of a kinase inhibitor to determine its effects on tolerance. *In vivo* tolerance to meperidine, morphine and fentanyl were fully reversed by the PKC or PKA inhibitors. However, *in vivo* tolerance to DAMGO was not reversed by PKC or PKA inhibition. The GRK inhibitor did not reverse the tolerance to meperidine, fentanyl or morphine but did reverse the tolerance to DAMGO. These results suggest that  $\mu$ -opioid receptor desensitization induced by low- and moderate-efficacy  $\mu$ -agonists is dependent on PKC and PKA activity while  $\mu$ -opioid receptor desensitization induced by the high-efficacy  $\mu$ -agonist, DAMGO, is dependent on GRK activity.



## II. Introduction

Opioid analgesics are the most widely used drugs for the management of moderate to severe pain. One of the main drawbacks to this class of drugs is the development of tolerance during chronic use; that is, a decrease in the analgesic effect during prolonged use of the drug. The mechanisms of tolerance to any opiate are multifaceted and not fully understood. We and others have proposed that MOR desensitization plays an important role in opioid tolerance (Bailey *et al.*, 2006; Bohn *et al.*, 2000; Zuo, 2005). MOR desensitization can occur in at least two ways, through phosphorylation by GRK and subsequent arrestin binding or by phosphorylation by the second messenger kinases, PKC and PKA. The systems currently thought to play a role in opioid tolerance include receptor down regulation, receptor desensitization via receptor uncoupling from the G-protein (involving increased expression of protein kinase A (PKA) and protein kinase C (PKC)) and receptor internalization (involving GRK phosphorylation) among others (Zuo, 2005).

Previous studies have suggested that an opioid's intrinsic efficacy determines its ability to develop tolerance and that the mechanism of tolerance to different efficacy opioids may vary (Arden JR, 1995; Selley *et al.*, 1998; Yabaluri *et al.*, 1997). High intrinsic efficacy agonists like DAMGO have been hypothesized to produce tolerance by causing receptor desensitization through activation of GRKs and subsequent binding of arrestins (Keith *et al.*, 1998; Zaki *et al.*, 2000; Zhang *et al.*, 1998). Low- and moderate- intrinsic efficacy agonists do not appear to produce tolerance by affecting  $\mu$ -opioid receptor density and mRNA levels but instead generate tolerance through second messenger signaling

pathways such as the PKC and PKA pathways (Selley *et al.*, 1998). It has previously been shown in mature brain neurons that  $\mu$ -opioid receptor desensitization due to morphine exposure is induced primarily through PKC- and PKA-dependent phosphorylation while receptor internalization due to DAMGO is induced through GRK-dependent phosphorylation (Bailey *et al.*, 2004; Bailey *et al.*, 2006; Johnson *et al.*, 2006; Kelly *et al.*, 2008).

The objective of this study was to investigate the effect of PKC, PKA and GRK inhibition *in vivo* on antinociceptive tolerance following repeated administration of opioid agonists of different efficacy. The opioid agonists tested *in vivo* had low- (meperidine), moderate- (morphine and fentanyl) and high-efficacy (DAMGO) at the  $\mu$ -opioid receptor as determined by GTP $\gamma$ S assays (Selley *et al.*, 1998; Selley *et al.*, 1997).

Our results demonstrate that inhibition of GRK causes reversal of DAMGO-induced tolerance but not meperidine-, morphine- or fentanyl-induced tolerance while the inhibition of PKC or PKA results in the reversal of meperidine-, morphine- and fentanyl-induced tolerance but not that induced by DAMGO.

These results show that the mechanisms of tolerance to opioid agonists are specific to the efficacy of the agonist. GRK-dependent internalization mechanisms play a more important role in DAMGO-induced tolerance while the desensitization of the receptor which is dependent on the PKC or PKA pathways plays a more important role in the tolerance induced by low- to moderate-efficacy opioids.

### III. Materials and Methods

#### IIIa. Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25-30 g were housed 6 to a cage in animal care quarters and maintained at  $22 \pm 2$  °C on a 12-h light-dark cycle. Food and water were available *ad libitum*. The mice were brought to a test room ( $22 \pm 2$  °C, 12 hr light-dark cycle), marked for identification and allowed 18 hr to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the IASP (International Association for the Study of Pain).

#### IIIb. Tail immersion test

The warm-water tail immersion test was performed according to Coderre and Rollman (Coderre *et al.*, 1983) using a water bath with the temperature maintained at  $56 \pm 0.1$  °C. Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 seconds were used. The test latency after drug treatment was assessed at 30 minutes for morphine, fentanyl, and meperidine and at 20 minutes for DAMGO, with a 10 seconds maximum cut-off time imposed to prevent tissue damage. Antinociception was quantified according to the method of Harris and Pierson (Harris *et al.*, 1964) as the percentage of maximum possible effect (% MPE) which was calculated as:  $\%MPE = [(test\ latency - control\ latency) / (10 - control\ latency)] \times 100$ . Percent MPE was calculated for each mouse using at least 6 mice per treatment group.

### IIIc. Intracerebroventricular Injections

Intracerebroventricular (icv) injections were performed as described by Pedigo et al. (Pedigo *et al.*, 1975). Mice were anesthetized with isoflurane and a horizontal incision was made in the scalp. A free-hand 5  $\mu$ l injection of drug or vehicle was made in the lateral ventricle (2 mm rostral and 2 mm lateral at a 45° angle from the bregma). The extensive experience of this laboratory has made it possible to inject drugs by this route of administration with greater than 95% accuracy. Immediately after testing, the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

### IIId. Model of *in vivo* acute opioid tolerance

An 8-hr antinociceptive tolerance model to morphine, fentanyl, and meperidine was developed as follows: Mice were injected subcutaneously (sc) once every 2 hours for 6 hours (total of 4 injections) with the minimum dose of opioid that produces maximum analgesia in naïve mice in the tail immersion test. Two hours after the final dose, mice were administered the kinase inhibitor or vehicle by intracerebroventricular (icv) injection and immediately challenged with various sc challenge doses of the opioid to construct dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. In a similar fashion, an 8 hour antinociceptive tolerance model to DAMGO was developed as follows: mice were injected icv once every hour (total of 8 injections) with the minimal dose of DAMGO which results in maximum analgesia in naïve mice in the tail immersion test. One hour after the final dose, mice were administered the inhibitor by icv injection and immediately challenged with various icv doses of DAMGO to construct dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. The icv morphine

tolerance model followed the same dosing scheme as DAMGO with the exception of a two hour time period between the final dose of icv morphine and the icv injections of inhibitor and the challenge dose of morphine. The above opioid doses producing maximum analgesia were chosen based on the construction of acute dose-response curves in naïve mice (data not shown).

### IIIe. Statistical Analysis

Opioid dose-response curves were generated for calculation of effective dose-50 (ED<sub>50</sub>) values using least-squares linear regression analysis followed by calculation of 95% confidence limits (95% C.L.) by the method of Bliss (Bliss, 1967). Tests for parallelism were conducted before calculating the potency-ratio values with 95% C.L. by the method of Colquhoun (Colquhoun, 1971) who notes that a potency-ratio value of greater than one, with the lower 95% C.L. greater than one, is considered a significant difference in potency between groups.

### III f. Drugs and chemicals

The PKC inhibitor, Gö6976 [12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)carbazole]; the PKA inhibitor myristoylated PKI (14–22) amide [PKI-(14-22)-amide; Myr-N-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH<sub>2</sub>]; the PKA inhibitor PKI 6-22 amide [Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH<sub>2</sub>]; the PKA inhibitor KT5720; and the GRK inhibitor,  $\beta$ -Adrenergic Receptor Kinase 1 ( $\beta$ -ARK 1 inhibitor; Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate) were purchased from Calbiochem (San Diego, CA, USA). The GRK inhibitor

Ro 32-0432 HCL (2-(8-[(Dimethylamino) methyl]-6,7,8,9-tetrahydropyridol[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide) was purchased from Sigma (St. Louis, MO, USA). Meperidine hydrochloride, fentanyl hydrochloride, morphine sulfate, and etorphine hydrochloride were obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). DAMGO ([D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin) was purchased from Tocris Bioscience, (Ellisville, MO, USA). Morphine, meperidine, and fentanyl were dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL, USA). DAMGO was dissolved in distilled water. PKI-(14-22)-amide and PKI 6-22 were dissolved in distilled water; the corresponding vehicle-injected mice were injected with distilled water.  $\beta$ -ARK 1 inhibitor, Gö6976, KT5720, Bisindoylmaleimide and Ro 32-0432 HCL were dissolved in 10% DMSO, 20% cremophor, 70% distilled water; the corresponding vehicle-injected mice were injected with 10% DMSO, 20% cremophor, and 70% distilled water. We have previously published on the use of this vehicle for icv injections (Smith *et al.*, 1999; Smith *et al.*, 2002; Smith *et al.*, 2003; Smith *et al.*, 2006). The selected doses of the PKA and PKC inhibitors were shown to reverse morphine tolerance in a 3-day morphine pellet tolerance model (Smith *et al.*, 1999; Smith *et al.*, 2002; Smith *et al.*, 2003; Smith *et al.*, 2006).

## **IV. Results**

### **IVa. Opioid antinociceptive tolerance**

Low-, moderate- and high-efficacy opioid agonists produced similar levels of antinociceptive tolerance, as measured by the tail immersion test. The repeated administration of the low-efficacy  $\mu$ -opioid agonist meperidine (40 mg/kg sc every 2 hours for a total of 4 injections with test doses of meperidine administered 2 hours after the last injection) resulted in 2.7-fold tolerance in the tail immersion test. Similarly, the repeated administration of the moderate-efficacy  $\mu$ -opioid agonists morphine (10 mg/kg sc every 2 hours for a total of 4 injections with test doses of morphine administered 2 hours after the last injection) and fentanyl (0.2 mg/kg sc every 2 hr for a total of 4 injections with test doses of fentanyl administered 2 hours after the last injection) resulted in a 4.6- and 3.5-fold antinociceptive tolerance, respectively. The repeated administration of DAMGO (25.7 ng/kg icv every 1 hour for a total of 8 injections with test doses of DAMGO administered 1 hour after the last injection) resulted in 2.4-fold antinociceptive tolerance (Table 1).

**Table 1: Opioid antinociceptive tolerance using an 8hr model**

<b>Treatment</b>	<b>ED<sub>50</sub> value (<math>\mu</math>g/kg (95% C.L.))</b>		<b>Potency Ratio (95% C.L.)</b>
Vehicle + Meperidine Meperidine + Meperidine	15.1 mg/kg (11.3, 20.1) 41.9 mg/kg (37.1, 47.3)	vs. Vehicle + Meperidine	2.73 (2.29, 3.23)
Vehicle + Fentanyl Fentanyl + Fentanyl	0.089 mg/kg (0.08, 0.1) 0.292 mg/kg (0.26, 0.33)	vs. Vehicle + Fentanyl	3.50 (3.09, 3.92)
Vehicle + Morphine Morphine + Morphine	3.5 mg/kg (2.9, 4.2) 16.3 mg/kg (13.4, 19.8)	vs. Vehicle + Morphine	4.55 (3.77, 5.69)
Vehicle + DAMGO DAMGO + DAMGO	11.3 ng/kg (10.3, 12.3) 28.3 ng/kg (25.7, 36.0)	vs. Vehicle + DAMGO	2.42 (2.13, 2.73)

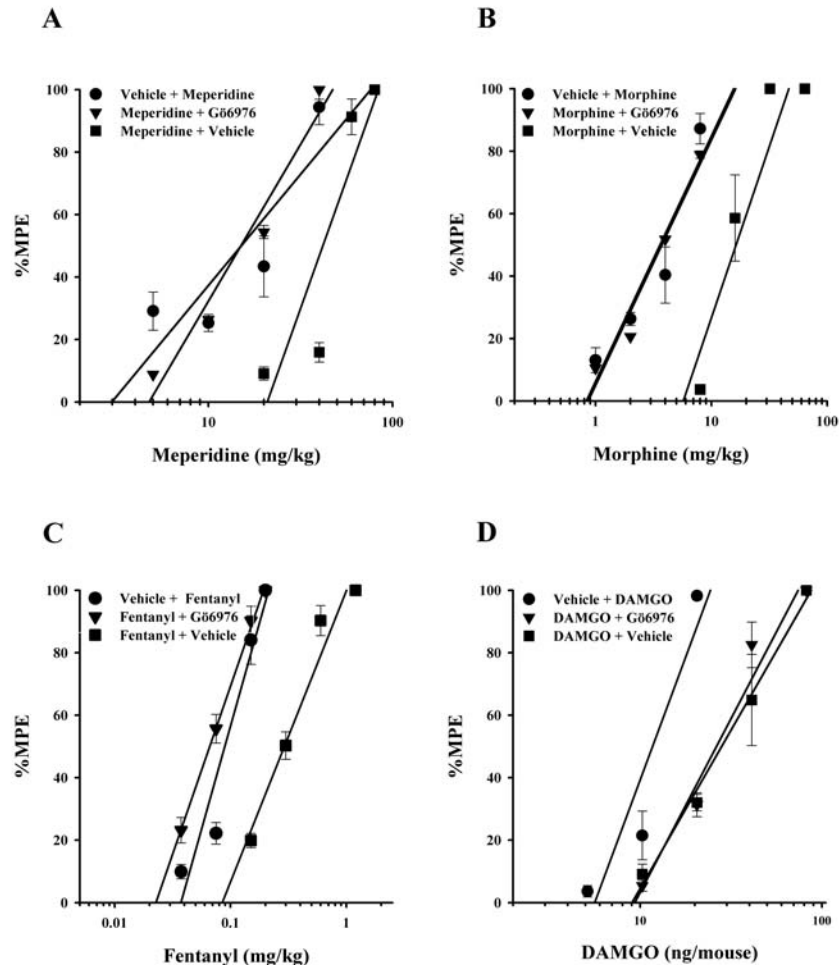
Mice were either repeatedly administered vehicle over 8 hours and then challenged with the opioid (Vehicle + Opioid) or repeatedly administered opioid over 8 hours and then challenged with the opioid (Opioid + Opioid). Meperidine (40 mg/kg sc), Morphine (10 mg/kg sc), and Fentanyl (0.2 mg/kg sc) were administered every 2 hours for a total of 4 injections with test doses of the opioid administered 2 hours after the last injection. DAMGO (25.7 ng/kg icv) was administered every 1 hour for a total of 8 injections with test doses of DAMGO administered 1 hour after the last injection. 30 minutes (20 minutes for DAMGO) after test doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.



#### IVb. Effects of PKC inhibition on opioid antinociceptive tolerance

The PKC inhibitor, Gö6976 (4 nmol/mouse; icv) administered to tolerant mice immediately before the challenge doses of opioid, fully reversed morphine, meperidine and fentanyl antinociceptive tolerance but did not reverse tolerance induced by DAMGO (Table 2 & Figure 1). Gö6976 produced a 100% reversal of the antinociceptive tolerance observed in mice repeatedly-administered the low-efficacy  $\mu$ -opioid agonist meperidine (Figure 1-A). Similarly, the antinociceptive tolerance observed in mice repeatedly-administered either of the moderate-efficacy  $\mu$ -opioid agonists, morphine or fentanyl, was fully reversed (99% and 100% reversal, respectively) by the administration of the PKC inhibitor Gö6976 (Table 2, Figures 1-B & 1-C). However, this same treatment with Gö6976 failed to significantly reverse the antinociceptive tolerance observed in mice repeatedly administered the high-efficacy  $\mu$ -opioid agonist DAMGO with only 12% reversal (Table 2, Figure 1-D).

**Figure 1: PKC inhibitor Go6976-induced reversal of low- and moderate-efficacy but not high-efficacy opioid tolerance**



Go6976 (4nmol/mouse icv) completely reversed antinociceptive tolerance in (A) meperidine-, (B) morphine- and (C) fentanyl-tolerant mice but not in (D) DAMGO-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes (20 minutes for DAMGO) after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc or icv for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve. ▼ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of the indicated opioid to construct a dose response curve. ■ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve.

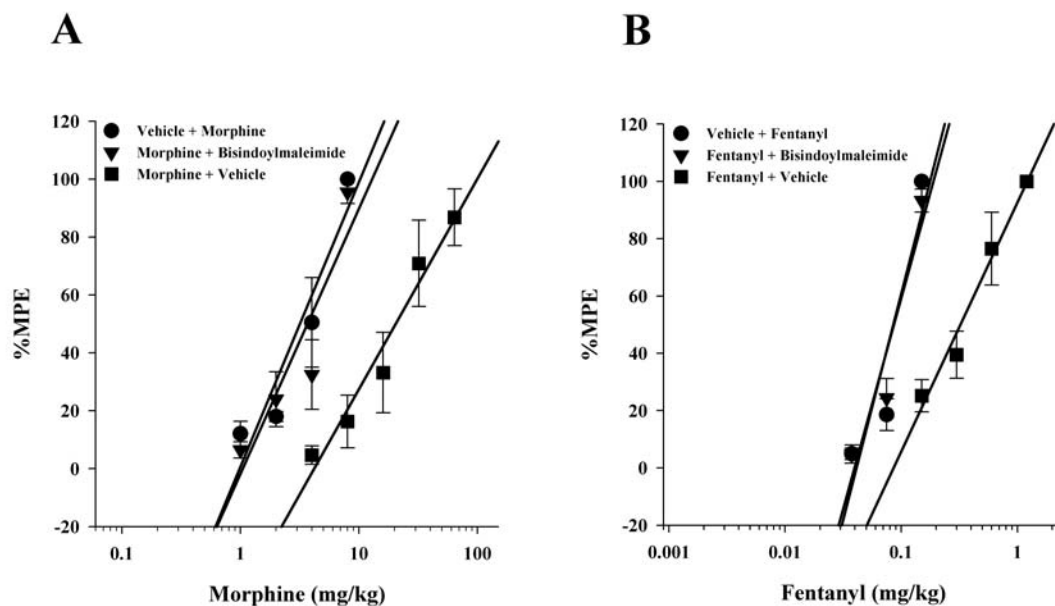
**Table 2: PKC inhibitor Gö6976**

<b>Treatment</b>	<b>ED<sub>50</sub> value (xg/kg (95%C.L.))</b>	<b>Potency Ratio (95% C.L.)</b>
Meperidine + Vehicle Meperidine + Gö6976	41.9 mg/kg (37.1, 47.3) 15.0mg/kg (12.9, 17.5)	vs. Meperidine + Vehicle 2.76 (2.41, 3.16)
Fentanyl + Vehicle Fentanyl + Gö6976	0.292 mg/kg (0.26, 0.33) 0.07mg/kg (0.06, 0.07)	vs. Fentanyl + Vehicle 4.65 (4.12, 5.26)
Morphine + Vehicle Morphine + Gö6976	16.3 mg/kg (13.4, 19.8) 3.6mg/kg (2.6, 4.8)	vs. Morphine + Vehicle 4.33 (3.30, 5.20)
DAMGO + Vehicle DAMGO + Gö6976	28.3 ng/kg (25.7, 36.0) 26.2 ng/kg (25.7, 30.8)	vs. DAMGO + Vehicle 1.07 (0.89, 1.30)

Mice were either repeatedly administered opioid over 8 hours and then administered vehicle icv immediately before being challenged with the opioid (Opioid + Vehicle) or repeatedly administered opioid over 8 hours and then administered the PKC inhibitor Gö6976 (4nmol/mouse icv) immediately before being challenged with the opioid (Opioid + Gö6976). Meperidine (40 mg/kg sc), morphine (10 mg/kg sc), and fentanyl (0.2 mg/kg sc) were administered every 2 hours for a total of 4 injections with inhibitor or vehicle and then test doses of the opioid were administered 2 hours after the last injection. DAMGO (25.7 ng/kg icv) was administered every 1 hour for a total of 8 injections with inhibitor or vehicle and then test doses of DAMGO were administered 1 hour after the last injection. 30 minutes (20 minutes for DAMGO) after test doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.

We also initially used the less specific PKC inhibitor bisindoylmaleimide HCL I to inhibit PKC in opioid tolerance (Figure 2). Bisindoylmaleimide HCL I fully reversed tolerance to morphine (100%) (Figure 2A) and fentanyl (99%) (Figure 2B).

**Figure 2: PKC inhibitor Bisindoylmaleimide-induced reversal of moderate-efficacy opioid tolerance**

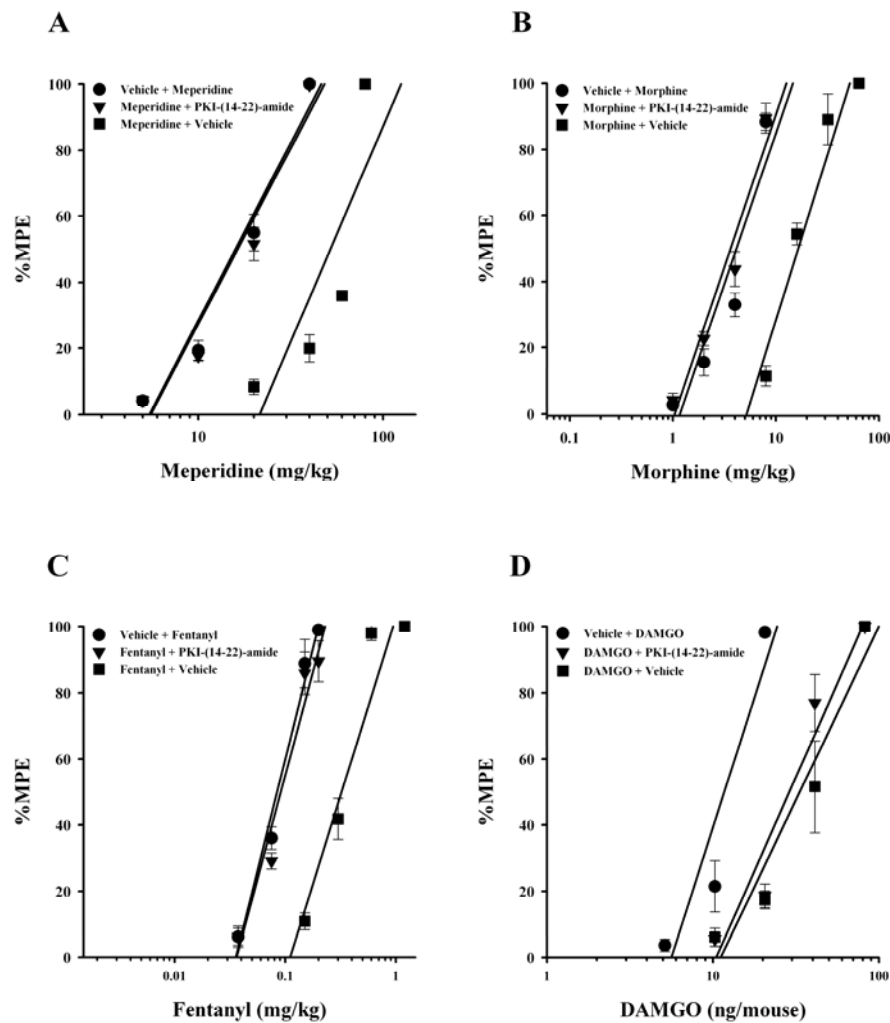


Bisindoylmaleimide HLC (55.5nmol/mouse icv) completely reversed antinociceptive tolerance in (A) morphine- and (B) fentanyl-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Vehicle + Opioid). ▼ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Bisindoylmaleimide). ■ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Vehicle).

#### IVc. Effects of PKA inhibition on opioid antinociceptive tolerance

The PKA inhibitor, PKI-(14-22)-amide (3.75 nmol/mouse; icv) fully reversed morphine, meperidine and fentanyl antinociceptive tolerance but did not reverse DAMGO-induced tolerance. As can be seen in Table 3 and in Figure 3A, the meperidine antinociceptive tolerance observed in mice repeatedly administered the low-efficacy  $\mu$ -opioid agonist meperidine was reversed 99% with the administration of PKI-(14-22)-amide. The antinociceptive tolerance observed in mice repeatedly administered the moderate-efficacy  $\mu$ -opioid agonists morphine (Figure 3B) or fentanyl (Figure 3C) was reversed 100% and 97% respectively with the administration of the PKA inhibitor PKI-(14-22)-amide. However, only 21% reversal of the antinociceptive tolerance was observed in mice repeatedly administered the high-efficacy  $\mu$ -opioid agonist DAMGO (Figure 3D).

**Figure 3: PKA peptide inhibitor (PKI) 14-22 amide induced reversal of low- and moderate-efficacy but not high-efficacy opioid tolerance**



PKI-(14-22)-amide (3.75 nmol/mouse icv) completely reversed antinociceptive tolerance in (A) meperidine-, (B) morphine- and (C) fentanyl-tolerant mice but not in (D) DAMGO-tolerant mice. Every data point represents an  $n=6$  of male Swiss Webster mice (25-30g). Tolerance was determined by the tail immersion test 30 minutes (20 minutes for DAMGO) after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc or icv for construction of dose-response curves for calculation of  $ED_{50}$  values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Vehicle + Opioid). ▼ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + PKI-(14-22)-amide). ■ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Vehicle).

**Table 3: PKA inhibitor PKI-(14-22)-amide**

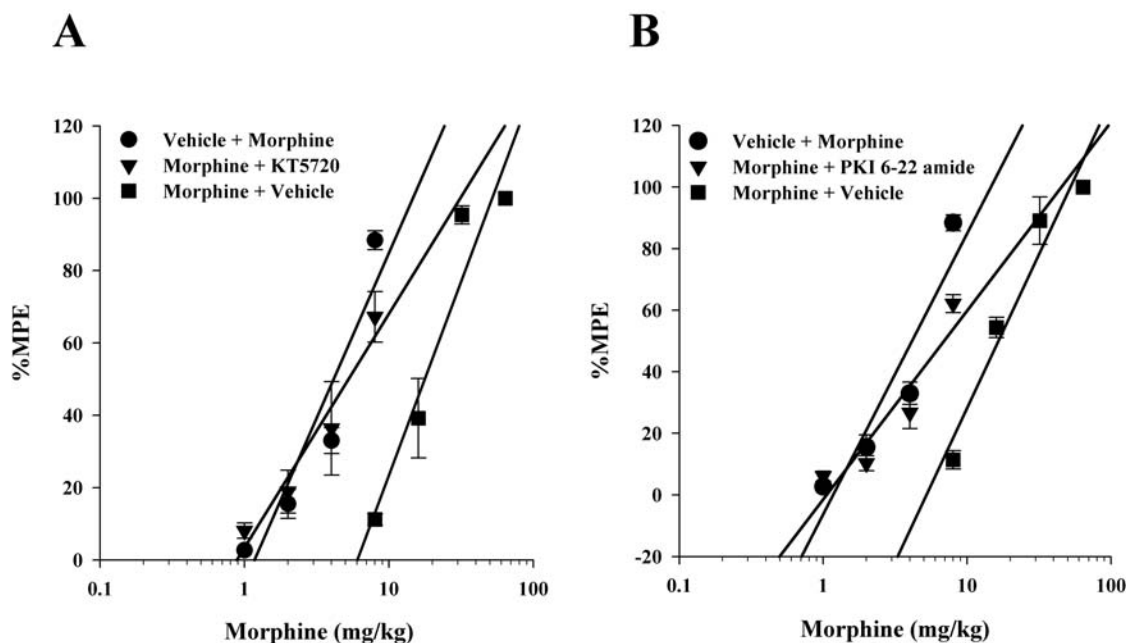
<b>Treatment</b>	<b>ED<sub>50</sub> value (xg/kg (95%C.L.))</b>	<b>Potency Ratio (95% C.L.)</b>
Meperidine + Vehicle	48.9 mg/kg (40.8, 58.7)	3.14 (95% C.L. 2.89, 3.43) vs. Meperidine + Vehicle
Meperidine + PKI-(14-22)-amide	16.3 mg/kg (14.8, 18.1)	
Fentanyl + Vehicle	0.338 mg/kg (0.3, 0.38)	3.62 (95% C.L. 3.23, 4.11) vs. Fentanyl + Vehicle
Fentanyl + PKI-(14-22)-amide	0.091 mg/kg (0.08, 0.1)	
Morphine + Vehicle	16.3 mg/kg (13.3, 20.1)	4.45 (95% C.L. 3.80, 5.44) vs. Morphine + Vehicle
Morphine + PKI-(14-22)-amide	3.7 mg/kg (3.2, 4.2)	
DAMGO + Vehicle	33.4 ng/kg (25.7, 41.1)	1.15 (95% C.L. 0.95, 1.38) vs. DAMGO + Vehicle
DAMGO + PKI-(14-22)-amide	28.8 ng/kg (25.7, 30.8)	

Mice were either repeatedly administered opioid over 8 hours and then administered vehicle icv immediately before being challenged with the opioid (Opioid + Vehicle) or repeatedly administered opioid over 8 hours and then administered the PKA inhibitor PKI 14-22 (3.75nmol/mouse icv) immediately before being challenged with the opioid (Opioid + Gö6976). Meperidine (40 mg/kg sc), morphine (10 mg/kg sc), and fentanyl (0.2 mg/kg sc) were administered every 2 hours for a total of 4 injections with inhibitor or vehicle and then test doses of the opioid were administered 2 hours after the last injection. DAMGO (25.7 ng/kg icv) was administered every 1 hour for a total of 8 injections with inhibitor or vehicle and then test doses of DAMGO were administered 1 hour after the last injection. 30 minutes (20 minutes for DAMGO) after test doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.



Two other PKA inhibitors were also tested initially, KT5720 (Figure 4A) and PKI 6-22 amide (Figure 4B). Similar to the results found in 72 hour morphine pelleted tolerance by Dalton et al 2005 (Dalton *et al.*, 2005) and in unpublished data from our lab where PKI 6-22 only partially reversed morphine tolerance while KT5720 fully reversed morphine tolerance, these inhibitors were able to reverse morphine tolerance 77% and 91% respectively.

**Figure 4: PKA inhibitors KT5720 and PKI 6-22 amide induced reversal of moderate-efficacy opioid morphine tolerance**



**(A)** KT5720 (2.5nmol/mouse icv) completely reversed antinociceptive tolerance in morphine-tolerant mice. **(B)** PKI 6-22 amide (5nmol/mouse icv) partially reversed antinociceptive tolerance in morphine-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of morphine to construct a dose response curve (Vehicle + Morphine). ▼ indicates animals treated with morphine repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of morphine to construct a dose response curve (Morphine + Inhibitor). ■ indicates animals treated with morphine repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of morphine to construct a dose response curve (Morphine + Vehicle).

#### IVd. Effects of GRK inhibition on opioid antinociceptive tolerance

As shown in Table 4 and illustrated in Figure 5, the GRK inhibitor,  $\beta$ -ARK 1 inhibitor (a specific GRK-2 inhibitor; (Iino *et al.*, 2002)) did not reverse morphine, meperidine or fentanyl antinociceptive tolerance but did fully reverse DAMGO tolerance. The ED<sub>50</sub> values for meperidine, morphine and fentanyl in the repeatedly opioid-treated mice given  $\beta$ -ARK 1 inhibitor (20 nmol/5 $\mu$ l; icv), immediately before the challenging doses, were not significantly different from those calculated in the repeatedly opioid-treated mice given vehicle and the tolerance was only reversed 14%, 12%, and 0% respectively (Table 4; Figure 5A, 5B & 5C).

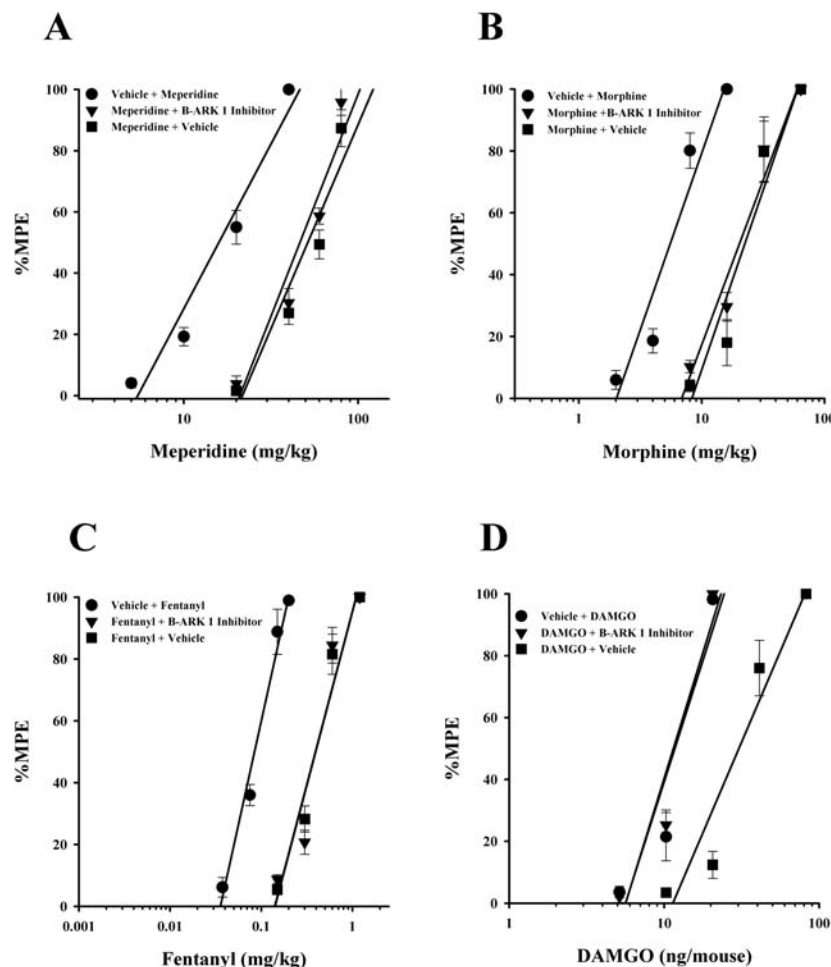
Interestingly, this same treatment with  $\beta$ -ARK 1 inhibitor fully (100%) reversed the antinociceptive tolerance observed in mice repeatedly administered the high-efficacy  $\mu$ -opioid agonist DAMGO (Figure 5D). The ED<sub>50</sub> value for DAMGO in the repeatedly DAMGO treated mice, given  $\beta$ -ARK 1 inhibitor immediately before the challenging doses, was significantly different from DAMGO tolerant mice given vehicle inhibitor (Table 4).

**Table 4: GRK inhibitor  $\beta$ -ARK 1 Inhibitor**

<b>Treatment</b>	<b>ED<sub>50</sub> value (<math>\mu</math>g/kg (95% C.L.))</b>	<b>Potency Ratio (95% C.L.)</b>
Meperidine + Vehicle	51.4 mg/kg (46.4, 56.9)	
Meperidine + $\beta$ -ARK 1	46.3 mg/kg (42.5, 50.5)	vs. Meperidine + Vehicle 1.10 (1.00, 1.21)
Fentanyl + Vehicle	0.392 mg/kg (0.35, 0.44)	
Fentanyl + $\beta$ -ARK 1	0.395 mg/kg (0.35, 0.45)	vs. Fentanyl + Vehicle 1.01 (0.90, 1.13)
Morphine + Vehicle	22.4 mg/kg (19.3, 26.1)	
Morphine + $\beta$ -ARK 1	20.3 mg/kg (17.6, 23.3)	vs. Morphine + Vehicle 1.10 (0.92, 1.32)
DAMGO + Vehicle	30.3 ng/kg (25.7, 36.0)	
DAMGO + $\beta$ -ARK 1	11.3 ng/kg (10.3, 15.4)	vs. DAMGO + Vehicle 2.57 (2.28, 2.88)

Mice were either repeatedly administered opioid over 8 hours and then administered vehicle icv immediately before being challenged with the opioid (Opioid + Vehicle) or repeatedly administered opioid over 8 hours and then administered the GRK inhibitor  $\beta$ -ARK 1 Inhibitor (20nmol/mouse icv) immediately before being challenged with the opioid (Opioid +  $\beta$ -ARK 1). Meperidine (40 mg/kg sc), morphine (10 mg/kg sc), and fentanyl (0.2 mg/kg sc) were administered every 2 hours for a total of 4 injections with inhibitor or vehicle and then test doses of the opioid were administered 2 hours after the last injection. DAMGO (25.7 ng/kg icv) was administered every 1 hour for a total of 8 injections with inhibitor or vehicle and then test doses of DAMGO were administered 1 hour after the last injection. 30 minutes (20 minutes for DAMGO) after test doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.

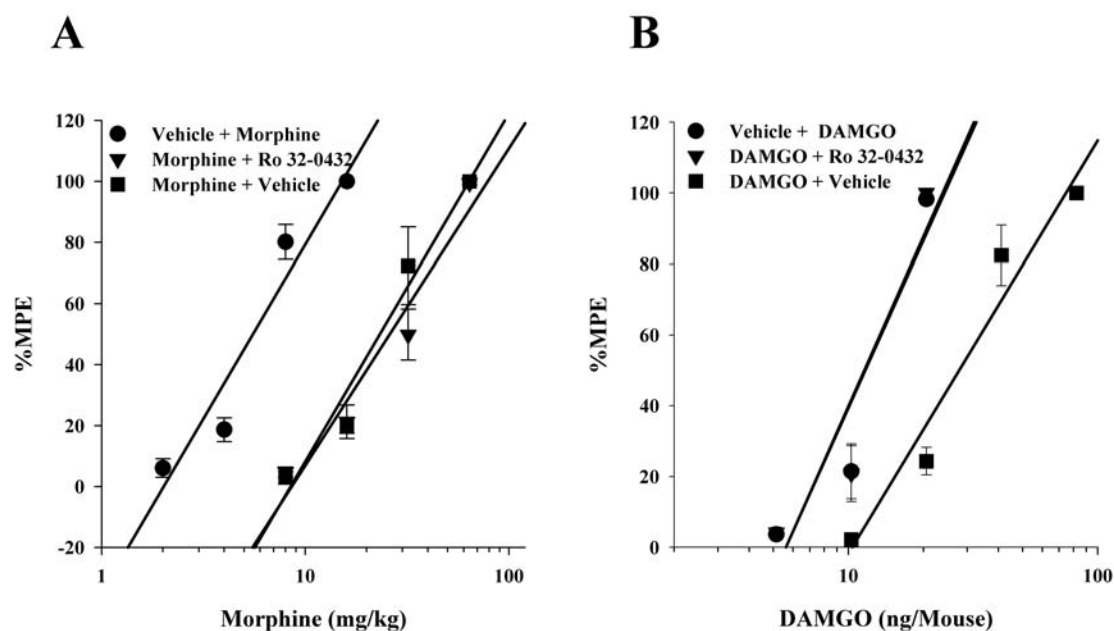
**Figure 5: G-protein coupled receptor kinase (GRK) inhibitor  $\beta$ -ARK 1 Inhibitor induced reversal of high-efficacy opioid but not moderate-efficacy opioid tolerance**



$\beta$ -ARK 1 Inhibitor (20nmol/mouse icv) completely reversed antinociceptive tolerance in **(D)** DAMGO-tolerant mice but not **(A)** meperidine-, **(B)** morphine- and **(C)** fentanyl-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes (20 minutes for DAMGO) after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc or icv for construction of dose-response curves for calculation of  $ED_{50}$  values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Vehicle + Opioid). ▼ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid +  $\beta$ -ARK 1 Inhibitor). ■ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Vehicle).

The GRK5 inhibitor, Ro 32-0432 (Ro 32-0432 is less specific than  $\beta$ -ARK 1 inhibitor as it also inhibits GRK 2 and 3 although to a lesser degree than it does GRK 5 (Aiyar *et al.*, 2000)) failed to reverse antinociceptive tolerance when administered icv (2 nmol/mouse) in mice repeatedly-administered morphine (Figure 6A). The ED<sub>50</sub> value for morphine in the repeatedly morphine-treated mice given Ro 32-0432 was not significantly different from that calculated in the morphine tolerant mice administered vehicle. However, this same inhibitor resulted in 97% reversal of antinociceptive tolerance in mice repeatedly-administered DAMGO (Table 5, Figure 6B).

**Figure 6: G-protein coupled receptor kinase (GRK) inhibitor Ro32-0432 induced reversal of high-efficacy opioid but not moderate-efficacy opioid tolerance**



Ro32-0432 (2nmol/mouse icv) completely reversed antinociceptive tolerance in **(B)** DAMGO-tolerant mice but not **(A)** morphine-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes (20 minutes for DAMGO) after the inhibitor or vehicle and opioid were administered, using various doses of the opioid sc or icv for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Vehicle + Opioid). ▼ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Ro 32-0432). ■ indicates animals treated with the indicated opioid repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of the indicated opioid to construct a dose response curve (Opioid + Vehicle).

**Table 5: GRK inhibitor Ro32-0432**

<b>Treatment</b>	<b>ED<sub>50</sub> value (xg/kg (95%C.L.))</b>	<b>Potency Ratio (95% C.L.)</b>
Morphine + Vehicle	23.2 mg/kg (20.0, 26.8)	
Morphine + Ro 32-0432	25.9mg/kg (22.6, 29.8)	vs. Morphine + Vehicle 1.11 (0.93, 1.33)
DAMGO + Vehicle	27.7 ng/kg (25.7, 30.8)	
DAMGO + Ro 32-0432	11.8 ng/kg (10.3, 15.4)	vs. DAMGO + Vehicle 2.34 (2.04, 2.65)

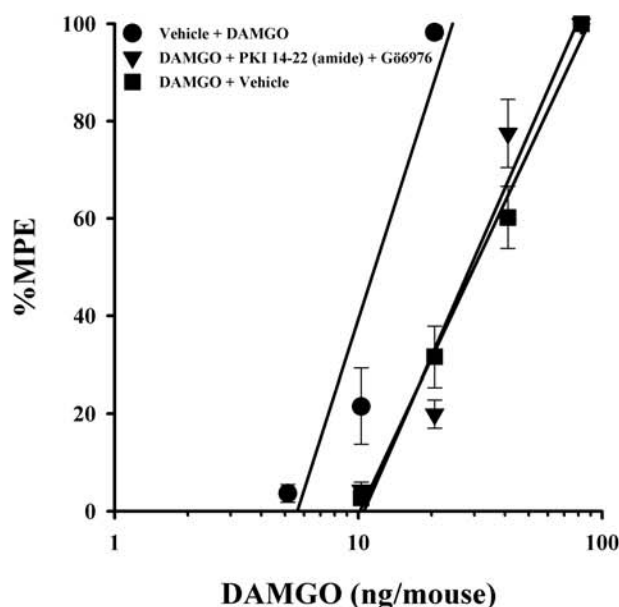
Mice were either repeatedly administered opioid over 8hrs and then administered vehicle icv immediately before being challenged with the opioid (Opioid + Vehicle) or repeatedly administered opioid over 8 hours and then administered the GRK inhibitor Ro 32-0432 (2nmol/mouse icv) immediately before being challenged with the opioid (Opioid + Ro 32-0432). Morphine (10 mg/kg sc) was administered every 2 hours for a total of 4 injections with inhibitor or vehicle and then test doses of morphine were administered 2 hours after the last injection. DAMGO (25.7 ng/kg icv) was administered every 1 hour for a total of 8 injections with inhibitor or vehicle and then test doses of DAMGO were administered 1 hour after the last injection. 30 minutes (20 minutes for DAMGO) after test doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.



#### IVe. Effects of combined administration of PKC and PKA inhibitors on DAMGO tolerance

In a previous study we showed that a higher level of tolerance to morphine (45-fold) was not fully reversed by very high doses of either a PKA inhibitor or a PKC inhibitor alone, but a full reversal was achieved by their combined administration (Smith *et al.*, 2003). To determine whether high-efficacy opioid tolerance, although of a lesser magnitude, was functionally similar to high levels of morphine tolerance, a PKA and a PKC inhibitor were administered simultaneously in DAMGO-tolerant animals. This combined treatment did not significantly reverse tolerance to DAMGO (6% reversal) (Figure 7).

**Figure 7: Combined inhibition of PKC (Gö6976) and PKA (PKI-(14-22)-amide) failed to reverse high-efficacy opioid tolerance**

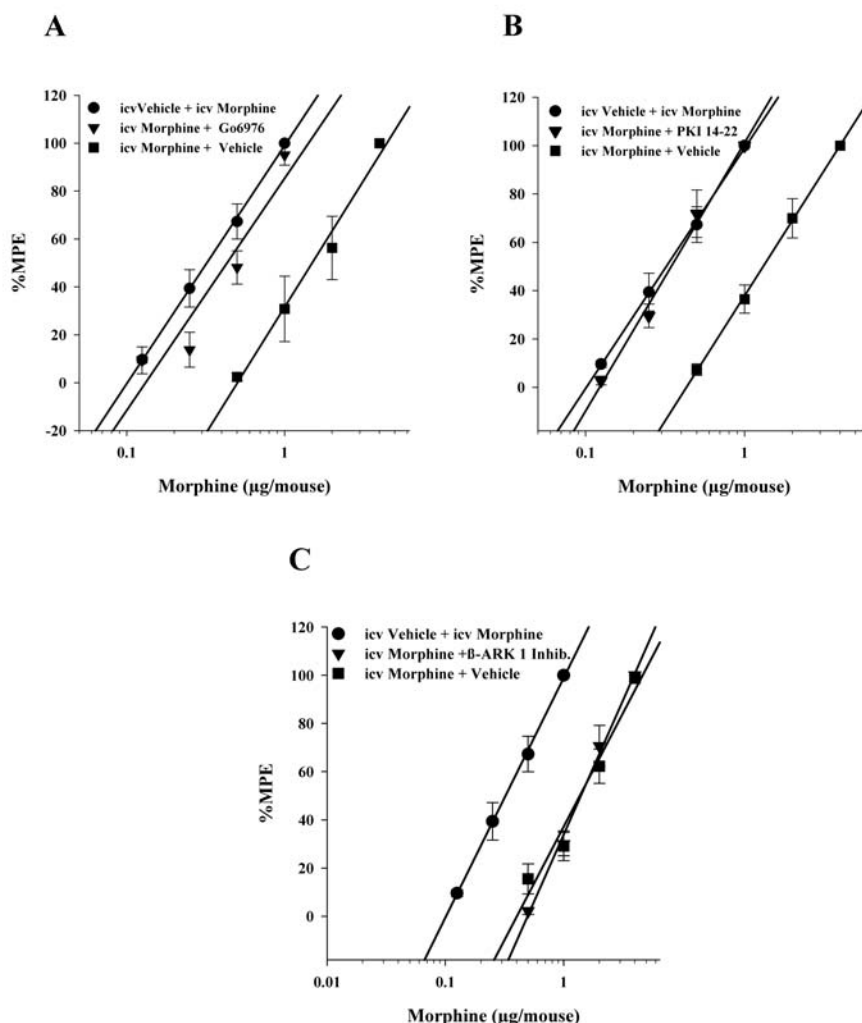


Gö6976 (4nmol/mouse icv) and PKI-(14-22)-amide (3.75 nmol/mouse icv) administered together failed to reverse antinociceptive tolerance in DAMGO-tolerant mice. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 20 minutes after the inhibitor or vehicle and DAMGO were administered, using various doses of DAMGO icv for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of DAMGO to construct a dose response curve (Vehicle + DAMGO). ▼ indicates animals treated with DAMGO repeatedly over 8 hours to build tolerance before being administered inhibitors icv and the challenge doses of DAMGO to construct a dose response curve (DAMGO + PKI-(14-22)-amide and Gö6976). ■ indicates animals treated with DAMGO repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of DAMGO to construct a dose response curve (DAMGO + Vehicle).

#### IVf. Morphine icv

It was important to address whether the differences in results between DAMGO and the other opioids was because of the difference in their efficacies or whether it had to do with the dosing scheme and route of administration. Morphine was administered in a similar route (icv) and dosing scheme as DAMGO and then the animals were administered either Go6976 (4 nmol/mouse; icv), PKI-(14-22)-amide (3.75 nmol/mouse; icv), or  $\beta$ -ARK 1 inhibitor (20 nmol/5 $\mu$ l; icv) and a challenge dose of morphine icv to construct a dose response curve. As can be seen in Figure 8 morphine tolerance was 94% reversed with the PKC inhibitor (Figure 8A) and 98% reversed with the PKA inhibitor (Figure 8B). However morphine tolerance was not reversed with the GRK inhibitor (Figure 8C). These results are the same as those in animals who were administered morphine sc (Figures 1B, 3B and 5B).

**Figure 8: Tolerance after the icv administration of morphine was reversed by the PKC inhibitor (Gö6976) and PKA inhibitor (PKI 14-22) but not by the (GRK) inhibitor  $\beta$ -ARK 1 inhibitor**



Gö6976 (4nmol/mouse icv) (A) or PKI 14-22 (3.75 nmol/mouse) (B) completely reversed icv morphine antinociceptive tolerance while  $\beta$ -ARK 1 inhibitor (20nmol/mouse) (C) failed to reverse icv morphine antinociceptive tolerance. Each data point represents 6 mice. Tolerance was determined by the tail immersion test 30 minutes after the inhibitor or vehicle and morphine were administered, using various doses of morphine sc or icv for construction of dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated only with vehicle for 8 hours before being administered vehicle icv and the challenge doses of morphine to construct a dose response curve (icv Vehicle + icv Morphine). ▼ indicates animals treated with morphine icv repeatedly over 8 hours to build tolerance before being administered inhibitor icv and the challenge doses of morphine icv to construct a dose response curve (icv Morphine + Inhibitor). ■ indicates animals treated with morphine icv repeatedly over 8 hours to build tolerance before being administered vehicle icv and the challenge doses of morphine icv to construct a dose response curve (icv Morphine + Vehicle).

## V. Discussion

There are several *in vitro* studies which have demonstrated that  $\mu$ -opioid receptor desensitization to high-efficacy opioids differs from that to low- and moderate-efficacy opioids (Bailey *et al.*, 2003; Johnson *et al.*, 2006) but these results have yet to be demonstrated for *in vivo* tolerance. To this end, the present study was aimed at investigating whether differences in tolerance to opioids of different agonist efficacy can be found *in vivo*. To test this hypothesis a rapid induction *in vivo* opioid tolerance model was developed using low-, moderate-, and high-efficacy opioids. Specific inhibitors for PKC, PKA and GRK were assessed for their ability to reverse opioid tolerance induced by the various opioids. We chose to study these three kinases because all three have been implicated in  $\mu$ -opioid receptor regulation and opioid tolerance.

In these experiments we demonstrated that antinociceptive tolerance can be developed over 8 hours with repeated injections of the low-efficacy  $\mu$ -opioid receptor agonist meperidine, the moderate-efficacy  $\mu$ -opioid receptor agonists morphine and fentanyl, and the high-efficacy  $\mu$ -opioid receptor agonist DAMGO. It is important to note that morphine and fentanyl, although of different potency, are of similar efficacy (Selley *et al.*, 1998; Selley *et al.*, 1997). Selley found no significant differences between the efficacy of morphine and fentanyl in multiple *in vitro* systems including the rat brain, mMOR-CHO cells and SK-N-SH cells.

Once tolerance to each of these opioids was established we found that the tolerance to meperidine, morphine and fentanyl were all fully reversed by the administration of a PKC

inhibitor or a PKA inhibitor but not by the administration of GRK inhibitors. However tolerance to DAMGO was reversed by the GRK inhibitors but not by a PKC inhibitor or a PKA inhibitor. We used two different GRK inhibitors; one (Ro 32-0432) inhibits GRK 5, 2, and 3 and the other ( $\beta$ -ARK 1 inhibitor) specifically inhibits GRK 2. Since both inhibited tolerance to DAMGO this suggests that GRK2 may be the GRK involved in regulating the  $\mu$ -opioid receptor. These results show that for DAMGO both desensitization of the  $\mu$ -opioid receptor and the development of tolerance *in vivo* involve GRK and not PKC or PKA.

In our studies we found that GRK2 inhibition did not reverse tolerance to either morphine or fentanyl *in vivo*. This is in partial agreement with (Terman *et al.*, 2004) who observed that, in GRK3 knockout animals (GRK2 knockout is lethal), morphine tolerance is unaffected but that tolerance to fentanyl was reduced in comparison to wild type animals. These subtle differences in GRK involvement in tolerance to different opioids may be due to the model used as well as to the specific GRK isoform investigated.

Our laboratory, as well as others, has previously reported on the involvement of PKC in mediating morphine tolerance and the ability of PKC inhibitors to reverse morphine tolerance (Bailey C.P. , 2006; Granados-Soto V., 2000). Granados-Soto demonstrated that rats infused with morphine for 5 days had significantly higher levels of PKC $\alpha$  and PKC $\gamma$  in the dorsal spinal horn. The higher levels of PKC $\alpha$  and PKC $\gamma$ , as well as the morphine antinociceptive tolerance, were prevented when the PKC inhibitor chelerythrine was co-infused with morphine during the 5-day treatment. In addition, it

was reported that PKC $\gamma$  null mutant mice exhibit significantly less tolerance than wild-type controls (Zeitz *et al.*, 2001). We previously reported that chemical inhibitors of PKC completely reversed morphine (Javed *et al.*, 2004) and prevented (Gabra *et al.*, 2008) antinociceptive tolerance in 72 hour morphine-pelleted mice challenged with sc morphine. We further showed that the injection of these PKC inhibitors reinstated antinociception, hypothermia and Straub tail in mice implanted with morphine pellets 72 hour earlier (Smith *et al.*, 2006).

Accumulating evidence also suggests an important role for increased PKA activity in the development of opioid tolerance. Shen et al demonstrated that icv administration of the anti-sense oligodeoxynucleotide to PKA mRNA blocked the antinociceptive tolerance to morphine in mice (Shen *et al.*, 2000). We previously reported that icv administration of the PKA inhibitor, PKI-(14-22)-amide completely reversed (Dalton G.D., 2005; Javed *et al.*, 2004; Smith *et al.*, 2003) and prevented (Gabra *et al.*, 2008) morphine antinociceptive tolerance in mice. We also demonstrated that PKA inhibition reinstated antinociception, hypothermia and Straub tail in mice implanted with morphine pellets 72 hour earlier (Smith *et al.*, 2006). Wagner et al 1998 found that in chronically morphine treated neurosecretory cells, from the hypothalamic arcuate nucleus, increased PKA activity was responsible for maintaining cellular tolerance to  $\mu$ -opioid receptor agonists and that activation of PKA mimicked the  $\mu$ -opioid receptor uncoupling similar to that seen in tolerance (Wagner *et al.*, 1998). The increase in PKA activity, along with up-regulation of cAMP levels, after prolonged opioid administration has been considered to be a cellular correlate for opioid tolerance (Sharma S.K., 1975; Wagner *et al.*, 1998).

GRKs have also been suggested to play a key role in  $\mu$ -opioid receptor desensitization and in tolerance to some opioids. GRKs phosphorylate agonist-activated G-protein coupled receptors (GPCRs) which results in recruitment of arrestins to the receptor leading to receptor uncoupling from G proteins and also to internalization for recycling or degradation (Lefkowitz, 1998; Pitcher *et al.*, 1998). Our collaborators in Bristol England have recently reported that, in both LC neurons and HEK 293 cells, rapid DAMGO-induced  $\mu$ -opioid receptor desensitization is GRK-mediated because it is blocked by over-expression of a GRK2 dominant negative mutant (Johnson *et al.*, 2006; Kelly *et al.*, 2008). GRK phosphorylation of the  $\mu$ -opioid receptor is not constant with each opioid agonist that activates the receptor. Exposure of HEK293 cells expressing the  $\mu$ -opioid receptors to morphine has been found to activate the receptor but not cause significant internalization (Johnson *et al.*, 2006; Zhang *et al.*, 1998). However when GRK was over expressed in HEK293 cells, morphine was able to cause internalization of the receptors through a GRK-dependent mechanism (Zhang *et al.*, 1998). In LC neurons and HEK293 cells DAMGO induces rapid  $\mu$ -opioid receptor desensitization (Bailey *et al.*, 2003). Also, in experiments designed to compliment those in this study our collaborators found that the GRK inhibitor  $\beta$ -ARK 1 Inhibitor significantly reversed DAMGO induced desensitization of the  $\mu$ -opioid receptors in LC neurons while the PKC inhibitor Gö6976 had no effect. Additionally *in vivo* opioid treatment has been found to lead to an up regulation of both GRKs and  $\beta$ -arrestin 2 (Chakrabarti *et al.*, 2001; Hurle, 2001). Using HEK-293 cells, in which GRK has been over expressed, Bohn et al were able to show



that this over-expression resulted in GRK playing a significant role in morphine-mediated  $\mu$ -opioid receptor desensitization (Bohn *et al.*, 2004).

One explanation for differences in kinase mechanisms in tolerance between opioid drugs is that the opioids induce different conformational changes of the receptor depending upon their efficacy. The ability of a G-protein coupled receptor, such as the  $\mu$ -opioid receptor, to acquire different conformations when activated by various ligands is referred to as functional selectivity (Kelly *et al.*, 2008; Mailman, 2007; Urban *et al.*, 2007). Opioid agonists would appear to stabilize distinct conformations of the receptor which are similar enough to allow the receptor to couple to the appropriate G-proteins to elicit similar downstream responses but unique enough to permit different desensitization mechanisms. Low- and moderate-efficacy opioids binding to the  $\mu$ -opioid receptor induce conformational changes which make the phosphorylation sites for PKC and PKA more readily available while high-efficacy opioids binding to the receptor causes conformational changes which make the phosphorylation sites for GRK more available thus differentiating the pathways for tolerance activated by the different opioids.

An alternate hypothesis resulting in different pathways being responsible for the tolerance of varying efficacy opioids is the different numbers of receptors hypothesized to be occupied to reach the same level of activation. High-efficacy drugs, by definition, are required to occupy significantly fewer receptors on a cell to illicit the same response as low- and moderate-efficacy drugs do by occupying a much larger percentage of the receptors. This is the basis of the calculation of intrinsic efficacy of a compound. By their

intrinsic efficacy it can be seen that while fentanyl is a more potent agonist than morphine it is not more efficacious (Lee *et al.*, 2003; Selley *et al.*, 1998; Selley *et al.*, 1997). A possible explanation for high-efficacy opioid tolerance being more reliant on GRK could be the lower number of receptors occupied in addition to the favorable conformational changes to the receptor. It is possible that GRK's role can only be predominant when a few receptors are occupied. If so, then any alteration to GRK's ability to internalize the receptor, leading to tolerance, would make a larger impact when a high-efficacy agonist is bound to only a small percentage of receptors. When a large enough percentage of receptors are occupied it is possible that PKC and PKA become predominate and overshadow GRK's involvement to such a degree that blocking GRK has no visible effect on the tolerance level. Similarly, when only a few receptors are occupied by high efficacy opioids, PKA and PKC involvement is unable to reach the critical amount needed to become a main component of tolerance. Therefore blocking their actions does not seem to alter the tolerance to high efficacy opioids.

Over-expressing the GRK's in cell systems has been shown to lead to the GRKs playing a significant role in morphine-mediated  $\mu$ -opioid receptor desensitization. Additionally, in cells which have been engineered to have an abnormally low level of GRKs, PKC was found to play a significant role in DAMGO mediated  $\mu$ -opioid receptor desensitization (Haberstock-Debic *et al.*, 2005). This shows that it is not an either/or difference in the mechanisms by which the different efficacy opioids generate tolerance, but a more complex interaction whereby one mechanism has prevalence over the other depending on

the agonist. It is possible that a combination of functional selectivity as well as the percentage of receptors occupied could be responsible for this complexity.

The ability of some  $\mu$ -opioid receptor agonists, DAMGO and etorphine for example, to induce GRK phosphorylation - thus recruiting arrestin and leading to internalization of the receptor - while others are unable to cause such recruitment and internalization (morphine for example) has been widely demonstrated *in vitro* (Keith *et al.*, 1996; Zhang *et al.*, 1998). In addition, it has been reported by Johnson that morphine and DAMGO induce desensitization of the  $\mu$ -opioid receptor through different mechanisms in human embryonic kidney (HEK) 293 cells expressing the rat  $\mu$ -opioid receptor 1 (MOR1) (Johnson *et al.*, 2006). Johnson and collaborators found that while both DAMGO and morphine stimulated GIRK currents and caused phosphorylation of the MOR1 and desensitization of the receptor, they did so by different mechanisms. They found that the phosphorylation of the receptor while occupied by DAMGO was due to GRK while morphine occupation of the receptor caused phosphorylation through PKC. It could be hypothesized that the alternate conformations of the receptor when occupied by the different efficacy agonists was responsible for these variations in the desensitization and internalization of the receptor.

One could argue that the difference we found between the low- and moderate-efficacy opioids and the high-efficacy opioid could be due to DAMGO being a peptide rather than due to its high efficacy. However, *in vitro* experiments have shown that there are only minor differences between DAMGO and other high-efficacy opioids in their stimulation

of G-proteins despite their structural differences (Saidak *et al.*, 2006). We did attempt to use etorphine in this rapid tolerance induction model; however, we were unable to develop significant tolerance.

Additionally, to address this issue of the hydrophilic opioids all being administered systemically while the only hydrophobic opioid had to be administered icv (in order to bypass the blood brain barrier), we administered one of the hydrophilic opioids, morphine, in a similar icv dosing scheme to the DAMGO treatment and then tested the ability of inhibitors to affect this morphine tolerance. We found no difference in the results of the inhibitors' actions on morphine tolerance whether morphine tolerance was developed through systemic or icv administration. This shows that the difference between the high-efficacy opioid tolerance reversal and the low- and moderate-efficacy opioids tolerance reversal is a result of the efficacy of the compounds and not the route of administration or dosing scheme.

It is also interesting to note that the administration of morphine icv further implicated the PAG as a major site of action in the tolerance we observed. In humans opioids are administered icv in occasions of extreme pain in cancer patients ((Lazorthes, 1988; Lazorthes *et al.*, 1988; Lenzi *et al.*, 1985; Lobato *et al.*, 1987; Sandouk *et al.*, 1991). It has been demonstrated that this application not only relieves pain that is difficult to treat through other routes of administration, but that the speed of onset of the opioid analgesia is very rapid. However, hydrophilic opioids such as morphine have a very slow permeation from the ventricular lumen and so the distribution of the drug by the time of

onset of its actions is very limited. This implies that the site of action must be very close to the site of administration. The PAG fits this requirement as it directly surrounds the ventricular space (Herz A, 1971; Tafani *et al.*, 1989).

In our experiments the administration of the hydrophobic opioid DAMGO and the inhibitors which were in the DMSO: Cremelphore: H<sub>2</sub>O vehicle icv could lead to wider distribution in the brain, and the systemically administered opioids likely also had a wide distribution throughout the brain making it more difficult to specify a specific region of action. However, the morphine administered icv and the inhibitors in the distilled water vehicle are less able to permeate the brain in the same manner. Given the replicated results of the actions of the inhibitors on morphine tolerance despite the route of administration of morphine lends credence to our use of icv injections to target a brain region which plays a vital role in opioid tolerance.

In conclusion, these results support our hypothesis that different mechanisms underlie tolerance to opioids of different efficacies. This is the first report of the ability of any PKC inhibitor to reverse *in vivo* tolerance to meperidine and fentanyl as well as the first *in vivo* reversal of DAMGO tolerance with a GRK inhibitor. A GRK-dependent mechanism plays a greater role in tolerance to high-efficacy opioids while a PKC-and PKA-dependent desensitization of the receptor mechanism plays a greater role in tolerance to low- and moderate-efficacy opioid agonists.

## CHAPTER 3

### The Role of CD38 in Morphine's Acute Actions and Tolerance

#### I. Summary

The intercellular signal transduction pathways which result in the antinociceptive effects of morphine as well as play a role in morphine tolerance have not yet been fully elucidated. It has been proposed that the fluctuation in the intracellular levels of  $\text{Ca}^{2+}$  is an important component of both the antinociception produced by morphine and morphine tolerance (Connor *et al.*, 1996; Hille, 1994; Tomura *et al.*, 1992; Yeo *et al.*, 2001). The multifunctional ectoenzyme CD38 regulates intracellular  $\text{Ca}^{2+}$  levels via the conversion of  $\text{NAD}^+$  into cADPR. cADPR then stimulates the ryanodine receptor on the endoplasmic reticulum to release  $\text{Ca}^{2+}$  into the cytosol. In this work we show that there is expression of the CD38 gene and active CD38 protein in the PAG tissue. Acutely, morphine's potency was increased by the *in vivo* application of CD38's substrate  $\beta\text{-NAD}^+$ . Further, morphine tolerance was reversed by three separate inhibitors of the CD38-cADPR-ryanodine receptor pathway. In experiments in  $\text{CD38}^{-/-}$  animals morphine was found to be less potent in antinociceptive assays as well as in measures of hypothermia and straub tail, but equipotent in locomotor activation assays compared to WT animals. Additionally, less tolerance was developed to morphine in the knockout animals compared to wild type animals.

## II. Introduction

Studies from this and other laboratories have demonstrated that morphine interacts with ryanodine-sensitive intracellular  $\text{Ca}^{2+}$  pools in the development of tolerance. Acute administration of high doses of the plant alkaloid ryanodine icv (i.e.,  $> 10 \mu\text{M}$ ; sufficient to block the ryanodine channel) prevented the inhibitory effect of  $\text{Ca}^{2+}$  on morphine analgesia (Smith *et al.*, 1995). Similarly, ryanodine blocked naloxone-precipitated withdrawal in morphine-dependent mice, and reversed antinociceptive tolerance in mice acutely challenged with morphine (Ohsawa *et al.*, 1999; Smith *et al.*, 1999).

Our hypothesis is that morphine antinociception is associated with alterations in the activity of CD38 ADP-ribosyl cyclase, leading to increases in cADPR.  $\text{Ca}^{2+}$  influx via voltage-sensitive  $\text{Ca}^{2+}$  channels has been demonstrated to stimulate ryanodine receptors resulting in dramatic increases in cytosolic  $\text{Ca}^{2+}$  through the phenomenon of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (CICR) (Solovyova *et al.*, 2002; Usachev *et al.*, 1997). Furthermore, cADPR has been demonstrated to bind ryanodine (RyR) receptors, and stimulate  $\text{Ca}^{2+}$  mobilization from endoplasmic reticulum pools in neurons. Three subtypes of RyR channels (RyR1, RyR2 and RyR3) are expressed in the central and peripheral nervous system (Furuichi *et al.*, 1994; Lokuta *et al.*, 2002; Mhyre *et al.*, 2000; Mori *et al.*, 2000) and *in vitro* studies indicate that RyR channel stimulation leads to the activation of physiological processes such as synaptic transmission and neurotransmitter release throughout the CNS (Reyes *et al.*, 1996; Shmigol *et al.*, 1995; Simpson *et al.*, 1996).

CD38 is a type II ectoenzyme glycoprotein that is capable of regulating intracellular  $\text{Ca}^{2+}$  homeostasis by converting  $\text{NAD}^+$  into cADPR (cADP-ribose) by ADP-ribosyl cyclase. It also produces ADPR and NAADP, both of which are also  $\text{Ca}^{2+}$  mobilizing molecules.

CD38 was first identified as a leukocyte differentiation antigen, and has subsequently been found in many tissues including the brain. Within cultured primary tissue from the brain, CD38 has been identified in both astrocytes cultured from the hippocampus and cortex, and hippocampal neurons (Ceni *et al.*, 2003; Hotta *et al.*, 2000; Verderio *et al.*, 2001). Confocal microscopic studies intended to detect immunofluorescent CD38, and co-stained with TUJ-1 antibodies (neuronal), revealed intense CD38 staining of the cell body plasma membrane and processes, while CD38 in astrocytes was diffusely distributed throughout the entire cell (Ceni *et al.*, 2003). A 110 kDa homodimer form of CD38 as well as higher oligomers have been found to form channels for transporting cADPR (Bruzzone *et al.*, 1998). As an ectoenzyme on the plasma membrane surface, CD38 can convert extracellular  $\text{NAD}^+$  into cADPR as well as function intracellularly in the same manner (De Flora *et al.*, 2004). The cADPR can, in turn, bind to ryanodine receptors leading to  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. The ectoenzyme capabilities of CD38 were revealed in cultured astrocytes and hippocampal neurons when it was demonstrated that adding  $\beta\text{-NAD}^+$  to the culture medium increased the levels of extracellular cADPR (Ceni *et al.*, 2003; Hotta *et al.*, 2000; Verderio *et al.*, 2001). However, neurons and astrocytes can transfer extracellular cADPR into intracellular sites through plasma membrane transporters (CNT2, CNT3, cs-csg, and CD38) (Nagai *et al.*, 2005) (Peng *et al.*, 2005). Neurons and astrocytes also possess Cx43 transporters that transport



intracellular NAD<sup>+</sup> to extracellular sites (De Flora *et al.*, 2004). Extracellular NAD<sup>+</sup> can then diffuse either in an autocrine or paracrine fashion to be converted into cADPR by the ectoenzyme CD38 ADP-ribosyl cyclase for intracellular signaling (De Flora *et al.*, 2004).

When human and murine CD38 were cloned in the early 1990s they were found to display an extracellular domain which had high sequence homology to the soluble *Aplysia* ADP-ribosyl cyclase (Harada *et al.*, 1993; Jackson *et al.*, 1990; States *et al.*, 1992). During the same time period it was also determined that the enzymatic function of *Aplysia* ADP-ribosyl cyclase was to produce cADPR (Glick *et al.*, 1991; Hellmich *et al.*, 1991; Lee *et al.*, 1991). The soluble *Aplysia* ADP-ribosyl cyclase has been crystallized and it has been determined that it displays a 4  $\alpha$ -helices “up-down bundle” which is a unique topology and is the major identifying characteristic of this super family (Prasad *et al.*, 1996; Schuber *et al.*, 2004). CD157 has also been crystallized and shows a global tridimensional structure which is very similar to that of the *Aplysia* cyclase and, interestingly, both crystallized as homo-dimers (Schuber *et al.*, 2004). CD38, however, was not easily crystallized like the other members of its superfamily. But because of the conserved amino acid sequence between the members of the family (approximately 30% similarity between CD38 and *Aplysia* ADP-ribosyl cyclase), as well as alignment of conserved cystein residues, a homology-based 3-dimensional structure has been proposed (Munshi *et al.*, 2000).

Although CD38 has been found on the cell surface there is also evidence that it is located

on internal membranes as well (Khoo *et al.*, 1999; Mizuguchi *et al.*, 1995; Sun *et al.*, 1999). It is possible that in addition to its being originally located at these internal sites, plasmamembrane bound CD38 may also be transferred to these internal membranes via its association with other cell-surface molecules (Funaro *et al.*, 1990). CD38 has been found in the membranes of rough ER, ribosomes, small vesicles, mitochondria, nuclear envelope as well as the plasma membrane and located on the cell bodies, axon terminals and postsynaptic densities (Yamada *et al.*, 1997). The Moutin lab also used confocal immunofluorescence microscopy in primary cultures of mouse hippocampal cells to confirm that CD38 is expressed in both neurons and glial cells, and they were also able to confirm the location of CD38 on both the plasma membrane as well as on intracellular membranes (Ceni *et al.*, 2003). The wide distribution in the cell and the variety of cell types in the brain where CD38 is found indicates that CD38 may play a number of signaling roles in neurons and astrocytes (Yamada *et al.*, 1997). In fact, the immunolabeling for CD38 on astrocytes found by Yamada et al was more intense than that found on the neurons, and was distributed throughout the astrocytes cell body and processes indicating that it plays a significant role in the actions of this cell type (Yamada *et al.*, 1997).

CD38 has also been found to be present not only in the adult but also at very early stages in development (Ceni *et al.*, 2003). Ceni et al found in C57B1/6J mice that as early as embryonic day 15, CD38 activity could be found and that the level of activity was similar to the levels found in postnatal day 1 brain samples which then increased greatly in the adult brain samples. They also identified CD38 generated ADPR as the main product in

all developmental stages which is to be expected from previously published findings on CD38's typical enzymatic functions. Additionally, they were able to identify CD38 as the enzyme responsible for the production of both cADPR and ADPR because in knockout animals neither  $\text{NAD}^+$  glycohydrolase nor GDP-ribosyl cyclase activity was found at any age (Ceni *et al.*, 2003).

It appears that all ADP-ribosyl cyclase activity in the brain is linked with CD38. First, in  $\text{CD38}^{-/-}$  knockout mice no ADP-ribosyl cyclase activity was detected, and second, cADPR levels were non-existent compared to wild-type control mice (Ceni *et al.*, 2003). A similar lack of activity has been detected in other tissues as well, indicating that CD38 is the major site of ADP-ribosyl cyclase activity in mice (Fukushi *et al.*, 2001).

Although CD38 forms three products it has only one substrate,  $\text{NAD}^+$ . There has been some debate about whether the formation of ADPR is through a direct hydrolysis of cADPR (referred to as the “sequential intermediate mechanism” or SIM) (Franco *et al.*, 2001; Kim *et al.*, 1993a; Sauve *et al.*, 1998) or if it is a separate competing reaction which uses  $\text{NAD}^+$  as a substrate with an ADP-ribosyl intermediate (referred to as the “partition mechanism” or PM). It has been determined that the formation of cADPR and ADPR are separate reactions and cADPR is not a substrate for the formation of ADPR; that the proposed PM mechanism is the actual mechanism. Experiments by Sauve et al used noncyclizable substrates  $\text{NMN}^+$  and nicotinamide-7-deaza-hypoxanthine dinucleotide (7-deaza NHD $^+$ ). They found that even with noncyclizable substrates CD38

was still able to rapidly hydrolyze both  $\text{NMN}^+$  and 7-deaza  $\text{NHD}^+$  indicating that a cyclization step was unnecessary for the hydrolysis reaction to occur (Sauve *et al.*, 1998).

When water is readily available to CD38 the enzyme shifts towards the production of cADPr as shown in site directed mutagenesis experiments in which residues in the active site of CD38 were mutated to allowed water greater access to the active site of the enzyme (Schuber *et al.*, 2004). The differences in the preference for the *Aplysia* ADP-ribosyl cyclase to generate a majority of cADPR and a minority of ADPR while the mammalian cyclases are the opposite (~97-99% of CD38's product is ADPR) (Howard *et al.*, 1993) therefore is most likely due to subtle differences in their ability to allow water into the active site. Additionally, there can be conformational changes in the existing proteins which would shift the production of one product over the other through associations with multiprotein complexes which might cause an increase in the rate of formation of the cyclic compound (Schuber *et al.*, 2004).

CD38 is also able to cyclize exogenous  $\text{NGD}^+$ , an  $\text{NAD}^+$  analog, into the fluorescent product cGDPR (Graeff *et al.*, 1994).  $\text{NGD}^+$  is actually a better substrate for the CD38's cyclization function than the endogenous  $\text{NAD}^+$ ; however it is a poor substrate for the hydrolysis function. Franco *et al* reported that the products generated by CD38 when given  $\text{NGD}^+$  were ~75% cGDPR and 25% GDPR instead of a majority of the hydrolysis product (which is the result when endogenous  $\text{NAD}^+$  is the substrate) (Franco *et al.*, 1998; Graeff *et al.*, 1994; Schuber *et al.*, 2004). The fact that  $\text{NGD}^+$  is very resistant to enzymatic hydrolysis led to the belief in the SIM mechanism. Due to the majority of

ADPR formed endogenously it was believed that CD38 performed the cyclization and hydrolysis functions in a stepwise manner as a sequential reaction mechanism, and that the high levels of cGDPR found when  $\text{NGD}^+$  was the substrate, was evidence of this because, they believed, CD38 was unable to hydrolyze cGDPR into GDPR. However CD38's cyclization and hydrolysis functions both use the  $\text{NAD}^+$  ( $\text{NGD}^+$ ) as substrate and are independent of each other, but dependent on how much water is available at the reaction site (PM mechanism). There are also reports that NADP is another endogenous substrate for CD38. The use of NADP as a substrate could result in alterations in the kinetics and stoichiometry of the CD38's enzymatic reactions (Berthelie *et al.*, 1998; De Flora *et al.*, 1997).

Previous work in our laboratory and in the laboratories of others has shown that one can alter both the acute and chronic effects of morphine by inhibiting or stimulating various steps in the signal transduction system of brain neurons. Since the 1960's it has been clear that manipulations of intracellular  $\text{Ca}^{2+}$  occur with morphine exposure and play an integral role in morphine induced antinociception. The mechanism of action of morphine is known to involve the phosphoinositol, adenylyl cyclase and GRK pathways. However their relative importance and order of activation are not fully understood. In addition to these enzymatic pathways investigated for their role in opioids' action we propose that the CD38-cADPR-ryanodine receptor pathway is involved in morphine's mechanism of action. Previous studies in our lab have shown that the ryanodine receptor and  $\text{Ca}^{2+}$  release from the ER play a role in morphine's actions (Smith *et al.*, 1999; Smith *et al.*, 1995) therefore it is a logical next step to examine the system which directly affects this

receptor via cADPR to continue to fully elucidate morphine's mechanism of action.

### **III. Materials and Methods**

#### IIIa. Animals

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN) weighing 25-30 g were housed 6 to a cage in animal care quarters and maintained at  $22 \pm 2$  °C on a 12 hour light-dark cycle. CD38-deficient mice (CD38<sup>-/-</sup>) (Cockayne *et al.*, 1998), backcrossed 12 generations to BALB/cBy mice, obtained from The Trudeau Institute Breeding Facility (Saranac Lake, NY), were also housed 6 to a cage in animal care quarters and maintained at  $22 \pm 2$  °C on a 12 hour light-dark cycle. Food and water were available *ad libitum*. The mice were brought to a test room ( $22 \pm 2$  °C, 12 hr light-dark cycle), marked for identification and allowed 18 hours to recover from transport and handling. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University Medical Center and comply with the recommendations of the IASP (International Association for the Study of Pain).

#### IIIb. Tail immersion test

The warm-water tail immersion test was performed according toCoderre and Rollman (Coderre *et al.*, 1983) using a water bath with the temperature maintained at  $56 \pm 0.1$  °C. Before injecting the mice, a baseline (control) latency was determined. Only mice with a control reaction time from 2 to 4 seconds were used. The test latency after drug treatment was assessed at 30 minutes, with a 10 seconds maximum cut-off time imposed to prevent

tissue damage. Antinociception was quantified according to the method of Harris and Pierson (Harris *et al.*, 1964) as the percentage of maximum possible effect (% MPE) which was calculated as:  $\%MPE = [(test\ latency - control\ latency) / (10 - control\ latency)] \times 100$ . Percent MPE was calculated for each mouse using at least 6 mice per treatment group.

### IIIc. Intracerebroventricular Injections

Intracerebroventricular (icv) injections were performed as described by Pedigo *et al.* (Pedigo *et al.*, 1975). Mice were anesthetized with isoflurane and a horizontal incision was made in the scalp. A free-hand 5  $\mu$ l injection of drug or vehicle was made in the lateral ventricle (2 mm rostral and 2 mm lateral at a 45° angle from the bregma). The extensive experience of this laboratory has made it possible to inject drugs by this route of administration with greater than 95% accuracy. Immediately after testing, the animals were euthanized to minimize any type of distress, according to IACUC guidelines.

### IIId. Statistical Analysis

Opioid dose-response curves were generated for calculation of effective dose-50 (ED<sub>50</sub>) values using least-squares linear regression analysis followed by calculation of 95% confidence limits (95% C.L.) by the method of Bliss (Bliss, 1967). Tests for parallelism were conducted before calculating the potency-ratio values with 95% C.L. by the method of Colquhoun (Colquhoun, 1971) who notes that a potency-ratio value of greater than one, with the lower 95% C.L. greater than one, is considered a significant difference in potency between groups.

### IIIe. Drugs and chemicals

Morphine sulfate was obtained from the National Institute on Drug Abuse (Bethesda, MD, USA). Morphine sulfate was dissolved in pyrogen-free isotonic saline (Hospira, Lake Forest, IL, USA). Nicotinamide,  $\beta$ -NAD<sup>+</sup>, NGD<sup>+</sup>, and 8-bromo-cADPR were all obtained from Sigma (St. Louis, MO). Ryanodine was obtained from BIOMOL (Enzo Life Sciences International Inc., Plymouth Meeting, PA). All were dissolved in distilled water.

### III f. Membrane Protein Preparation

Two PAG regions from each treatment group were pooled. Lysis buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0) and Complete Protease Inhibitor tablet (Roche Diagnostics Corporation, Indianapolis, IN) was added to the tissue (750  $\mu$ L). The tissue was sonicated for 3x10 sec bursts with resting on ice in between each burst. Each sample was centrifuged at 3000 rpm at 4°C for 15 minutes. The supernatant was removed and centrifuged at 50000 x g for 15 minutes at 4°C. The resulting pellet was resuspended in lysis buffer (without protease inhibitor) and incubated on ice for 1 hour. Final protein sample was aliquoted and stored at -80°C. Bradford (Bio-Rad, Hercules, CA) reagent was used to quantify protein content.

### III g. Western Blot Procedure

100  $\mu$ g of protein from each sample was brought to 100  $\mu$ L with sample buffer containing  $\beta$ -mercaptoethanol (20% v/v) and 50 mM dithiothreitol (added fresh just before use). The samples were boiled for 5 minutes and 25  $\mu$ L/lane was loaded onto a 10% Criterion gel (Bio-Rad, Hercules, CA). The gel was run at 200V until the dye front



just ran off the gel. Proteins on the gel were transferred to PVDF membrane (90V for 30 min). The PVDF membrane was rinsed briefly in 1xPBS and then incubated for 1 hour at room temperature in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE). The buffer was removed and CD38 primary antibody (M-19 from Santa Cruz, Santa Cruz, CA) in Odyssey blocking buffer (1:750 dilution) was added. The membrane was incubated overnight on at rocker at 4°C. Primary antibody was removed and the membrane was washed 3 x 5min with 1xPBS. A 1:5000 dilution of appropriate Odyssey secondary antibody (LI-COR Biosciences, Lincoln, NE) was added and the membrane was incubated for 1 hour at room temp. The secondary antibody was removed and the membrane was briefly rinsed in PBS. The membrane was then allowed to dry in the dark at room temp. Once the membrane was dry, it was scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) to quantify the amount of protein. The loading control protein,  $\alpha$ -tubulin, was also quantified to ensure proper loading of the gel.

#### IIIh. 72 hour Morphine Tolerance Model

A 75mg morphine or placebo pellet was implanted according to Way et al. (Way *et al.*, 1969). Mice were anesthetized with 2.5% isoflurane before shaving the hair around the base of the neck. The skin was cleansed with 10% providone iodine (General Medical Corp., Prichard, WV) and rinsed with alcohol before making a 1cm horizontal incision at the base of the neck. The underlying subcutaneous space toward the dorsal flanks was opened using a sterile glass rod. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision and subcutaneous space. A

placebo pellet or 75 mg morphine pellet was inserted in the space before closing the site with Clay Adams Brand, MikRon® AutoClip®9mm Wound Clips (Becton Dickinson and Co., Sparks, MD) and again applying iodine to the surface. The animals were allowed to recover in their home cages where they remained throughout the experiment.

### IIIi. Hot-Plate Test

The hot-plate test was performed as described by O'Callaghan and Holtzman (O'Callaghan *et al.*, 1975). The mice were placed on a Syscom Model 35D hot plate set at 55°C to obtain baseline latencies before drug administration. The mice were observed for licking either their fore- or hind limb or jumping in response to the heat. The baseline latencies ranged between 5 and 6 seconds. Testing occurred 30 minutes after morphine sc. A 30 second cut-off was employed to prevent tissue damage. Antinociception was quantified according to the method of Harris & Pierson (Harris *et al.*, 1964) as the percentage of maximum possible effect (% MPE) which is calculated as:  $\%MPE = [(test\ latency - control\ latency) / (30 - control\ latency) - 1] \times 100$ . Percent MPE was calculated for each mouse.

### IIIj. Straub Tail

The mice were observed for the development of Straub tail 30 minutes after administration of morphine sc. The Straub tail reaction was graded using the numerical scoring system of Kameyama et al. (1978) as follows; 0=0°, 0.5=1° to 30°, 1.0=31° to 45°, 1.5=46° to 60°, 2.0=61° to 90°, 2.5=more than 90°. The angle was measured above the horizontal plane of the table.

### IIIk. Hypothermia

Baseline rectal temperatures were obtained before exposure to morphine. Test rectal temperatures were obtained 30 minutes after morphine administration and the Change in Body Temperature ( $\Delta T_b$ ) was calculated.

### IIIl. HPLC analysis

#### *IIIl.a. PAG tissue sample Homogenation*

Tissue was flash frozen and 400ul of HEPES buffer (containing 10mM HEPES, 148mM NaCl, 5mM KCl, 1.3mM  $\text{CaCl}_2$ , 0.3mM  $\text{MgCl}_2$  and 5.5mM glucose (pH7.0), before use 1 tablet of Complete Proteinase Inhibitor Cocktail (Roche Diagnostics Corporation, Indianapolis, IN) was added to 40ml of HEPES) was added to the sample and was homogenated at ~1500rpm with 3 strokes, then the sample was sonicated for 10 seconds with a sonifier cell disrupter (model 185 Bransen) at 4°C at setting 8. Sample was then diluted 1:6 in HEPES and the protein concentration was determined using the Bradford assay.

#### *IIIl.b. Cyclase reaction*

The sample was diluted in HEPES to the required concentration and incubated with the required concentration of  $\text{NGD}^+$  in a 100ul reaction volume. The sample was then incubated for the required time at 37°C (30 minutes for all samples not optimizing for time). The reaction mixture was then centrifuged at 4°C through an Amicon microultrafilter at 13,800g for 15 minutes to remove proteins, transferred to glass auto-injection vials and analyzed by HPLC-fluorescence without further workup.

### *IIIc. HPLC Analysis*

Chromatography was accomplished with an HPLC system equipped with a solvent delivery system (model 1090L, Hewlett-Packard, Avondale, PA) and a photodiode array detector detecting the excitation wavelength of 300nm and the emission wavelength of 412nm (model 1040A, Hewlett-Packard) was used to detect the fluorescent products. Nucleotides were resolved on a 3 $\mu$ m Supelcosil LC-18 column (4.6 x 150mm) with a 5 $\mu$ m Supelcosil LC-18 guard column (4.6 x 20mm; Supelco, Bellefonte, PA). The injection volume was 20 $\mu$ l and the flow rate was 0.8ml/minute. The cGDPR peak identity was confirmed by the consistence in retention time with the cGDPR standard (~2.3 minutes). Peak Area data was collected and analyzed with a Hewlett-Packard Chemstation as described previously by Li et al. (Li *et al.*, 1998).

The mobile phase consisted of 150 mM ammonium acetate (pH 5.5) containing 5% methanol (solvent A) and 50% methanol (solvent B). The flow rate was 0.8 ml/min. Peak identities were confirmed by co-migration with known standards. Quantitative measurements were performed by comparison of known concentrations of standards.

The running steps were as follow:

0.01 Minutes	100% Solvent A	0% Solvent B
4.00 Minutes	100% Solvent A	0% Solvent B
12.00 Minutes	75% Solvent A	25% Solvent B
16.00 Minutes	0% Solvent A	100% Solvent B
20.00 Minutes	0% Solvent A	100% Solvent B
25.00 Minutes	100% Solvent A	0% Solvent B

### III m. Qualitative Real time PCR

Applied Biosystems SYBR Green kit or TagMan kit with the ABI Prism 7900HT Sequence Detection System including instruments and software were used for these experiments. The primers for 18S and CD38 were chosen using ABI primer design software. The thermal profile for one step RT-PCR using SYBR Green kit was as follows: 48°C for 30 minutes and 95°C for 10 minutes; then 40 cycles of 95°C for 15 seconds, 45°C for 1 minute. Dissociation detection was performed after each run to verify proper primer concentration and possible dimer formation. Primers for 18S (152 bp product) are: (forward) 5'GCGCTAGACTCCGAGAACAT-3' and (reverse) 5'-TGGCCACTTACTACCTGACCCTT-3'. Primers for CD38 (137 bp product) are: (forward) 5'-TTCTGGTCAGTTTGCTCTGG-3' and (reverse): 5'-CACGGAGGTGACTGGACTAA-3'.

#### *III ma. Experiments and data analysis*

The experiment run consisted of three reactions for each sample: a non-template control, 18S primer plus RNA as internal standard and detected gene primer plus RNA. Each reaction and its duplicate were 10µl and the same thermal profile as those determined in standard curve construction were used. Once  $C_T$  was obtained, the relative levels of target RNA ( $T_n$ ) normalized to an internal reference, 18S was calculated with the following equation:

$T_n = 2^{-\Delta\Delta C_T}$ ; where,  $\Delta\Delta C_T = \Delta C_{T,e} - \Delta C_{T,c}$  (e: experimental RNA; c: control RNA)

$\Delta C_T = C_{T,X} - C_{T,R}$  (the difference in  $C_T$  between target gene (X) and internal reference (R)).

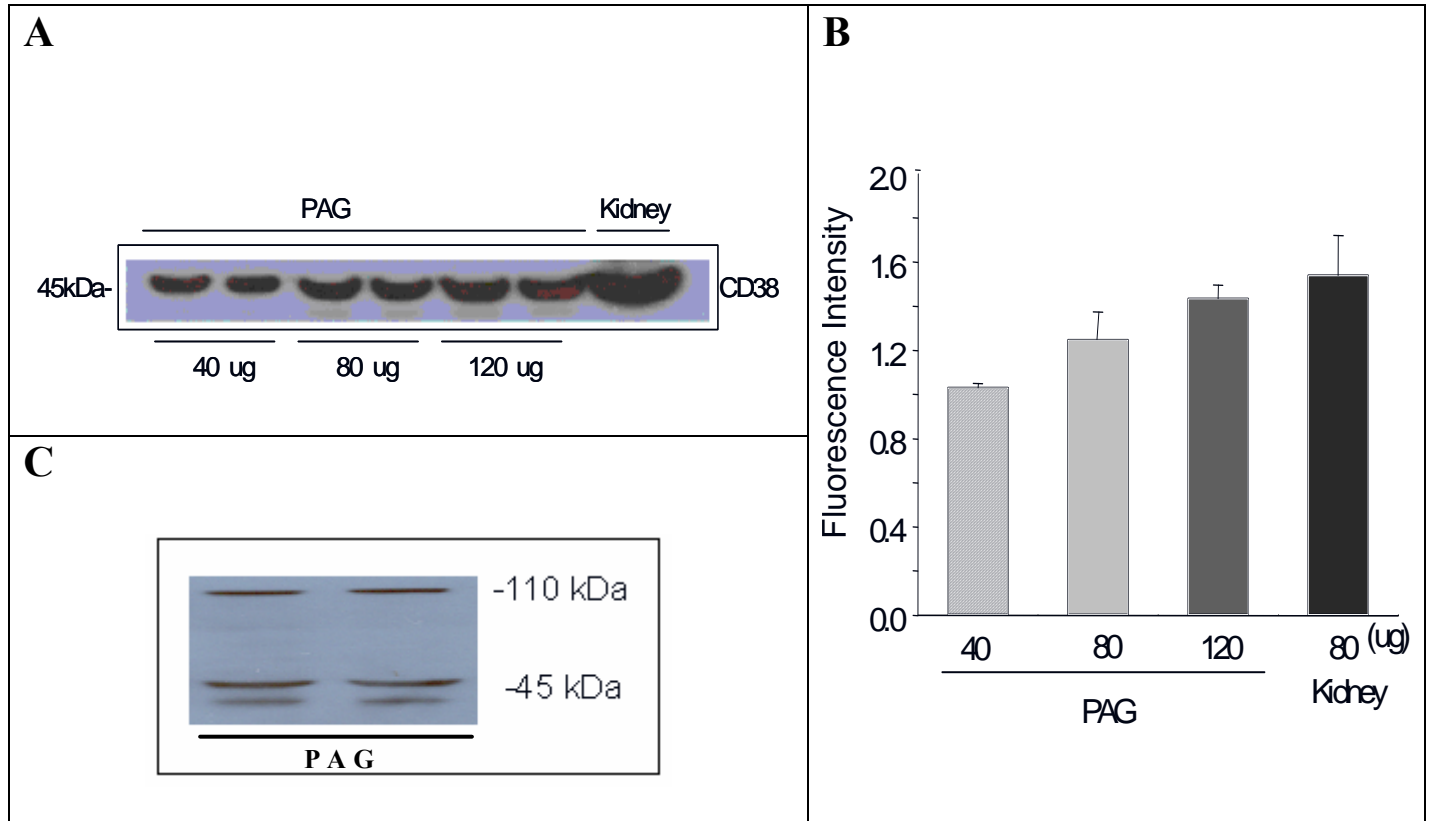
#### IV. Results

Previous work in our laboratory and in the laboratories of others have shown that one can alter both the acute and chronic effects of morphine by inhibiting or stimulating various steps in the signal transduction system of brain neurons. The overall goal of these series of experiments was to test the hypothesis that manipulating CD38 would have an effect on morphine's acute effects as well as morphine tolerance.

Our first objective was to quantify the level of CD38 protein in the periaqueductal grey (PAG) of the mouse brain. This area was chosen due to the high level of  $\mu$ -opioid receptors and the large amount of data in the literature showing the importance of this brain area in opiate-induced antinociception (Aston-Jones *et al.*, 1991; Reichling *et al.*, 1988). Varying protein concentrations from the PAG were separated by SDS-PAGE and transferred to a PVDF membrane for immunoblotting. CD38 levels were determined by immunoreaction with a specific CD38 antibody. The data presented in Figure 1A and 1B show that the amount of CD38 in the mouse PAG is comparable to that found in the kidney. The homodimer of CD38 has a molecular weight of around 110 kDa while the monomer is approximately 45kDa. The data presented in Figure 1C shows that both the monomer and homodimer form of CD38 are found in the PAG. In mice the 110 kDa homodimer on the plasma membrane surface can result from interdisulfide bonds at Cys123 and Cys205 between two 45 kDa monomers (Han *et al.*, 2002). Homodimers have been found in the plasma membrane in a variety of tissues including the brain (Khoo and Chang, 2000; Chidambaram *et al.*, 1998; Moreno-Garcia *et al.*, 2004) and are

known to both make and transport cADPR into the cell (Bruzzone et al., 1998; Moreno-Garcia et al., 2004).

**Figure 1: CD38 protein is present in the PAG**



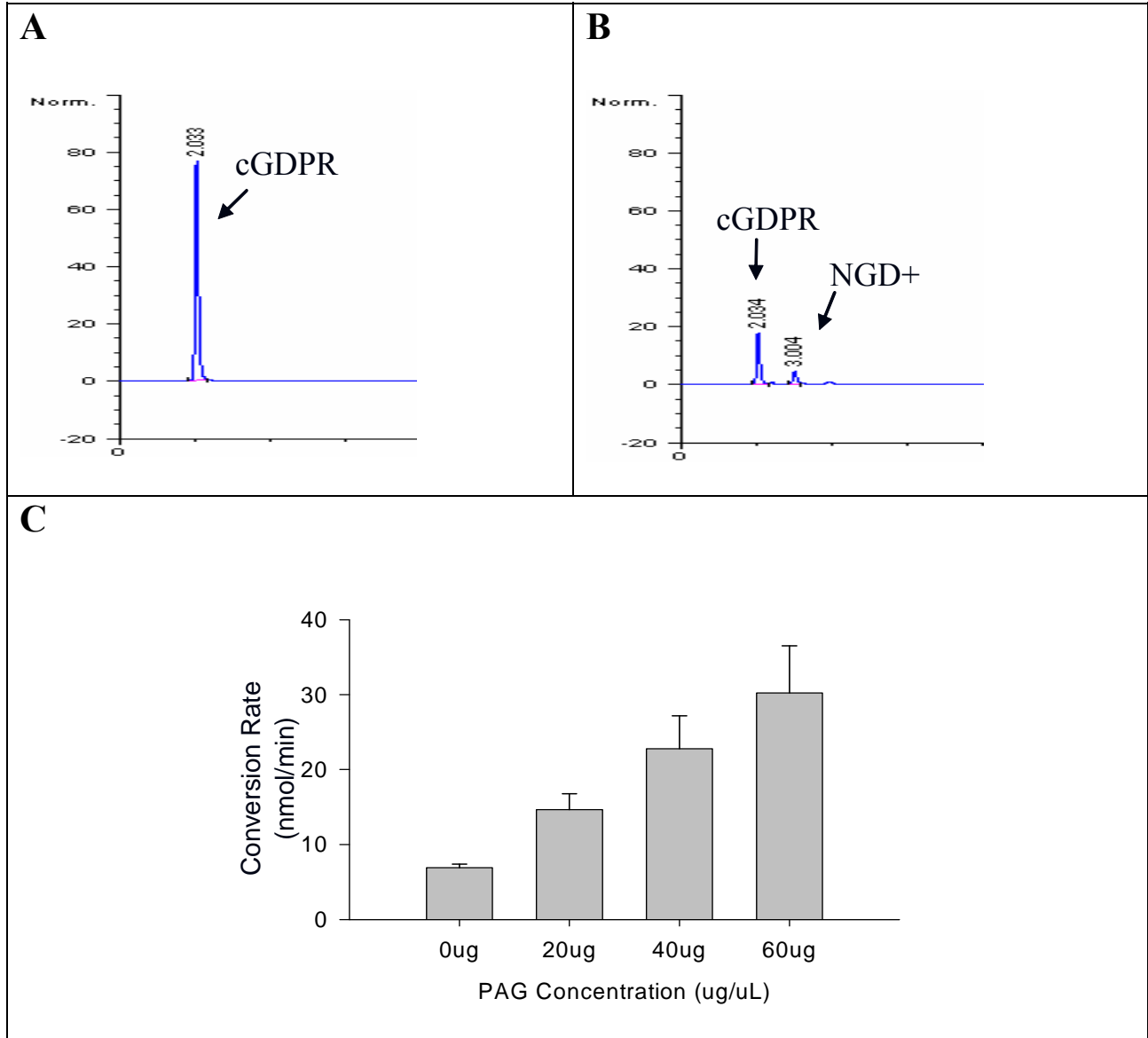
**(A)** Western immunoblot of CD38 protein in the PAG and kidney. Mice were sacrificed and tissue from mouse periaqueductal grey and kidney were removed and processed on a 10% gel and electrophoretically transferred onto PVDF. After blocking, the membrane was incubated with a 1:750 dilution of the CD38 primary antibody overnight at 4°C. The free primary antibodies were completely removed by washing with 1xPBS, and subsequently, the membrane was incubated for 1h with a 1:5000 dilution of the Odyssey secondary antibody. After washing the membrane to remove free secondary antibody the density of the blots was determined using the Odyssey Infrared Imaging System. **(B)** Quantitative comparison of the levels of CD38 in the PAG vs. Kidney reveals that the levels of CD38 protein in the PAG are comparable on a microgram basis to those in the kidney. **(C)** Both the 45 kDa monomer form and the 110 kDa dimer form of CD38 were found in the PAG tissue samples.



We next investigated whether there was gene expression of CD38 in mouse PAG. Quantitative real time-PCR (QRT-PCR) was used to show that CD38 mRNA was present in mouse PAG (data not shown).

*In vivo* CD38 naturally cyclizes  $\text{NAD}^+$  to cADPR. Using HPLC coupled with fluorescent detection to measure the amount of fluorescent cGDPR CD38 cyclizes from  $\text{NGD}^+$  the catalytic activity of CD38 can be quantitated (Sauve *et al.*, 2002). As seen in Figure 2B,  $\beta\text{-NGD}^+$  added to 40ug of PAG, led to a significant formation of cGDPR. Furthermore, as expected the assay was concentration dependent, in that 20 to 60  $\mu\text{g}$  PAG protein resulted in progressive increases in cGDPR formation, indicating that CD38 activity can be stimulated by the addition of  $\beta\text{-NGD}^+$  (Fig. 2C).

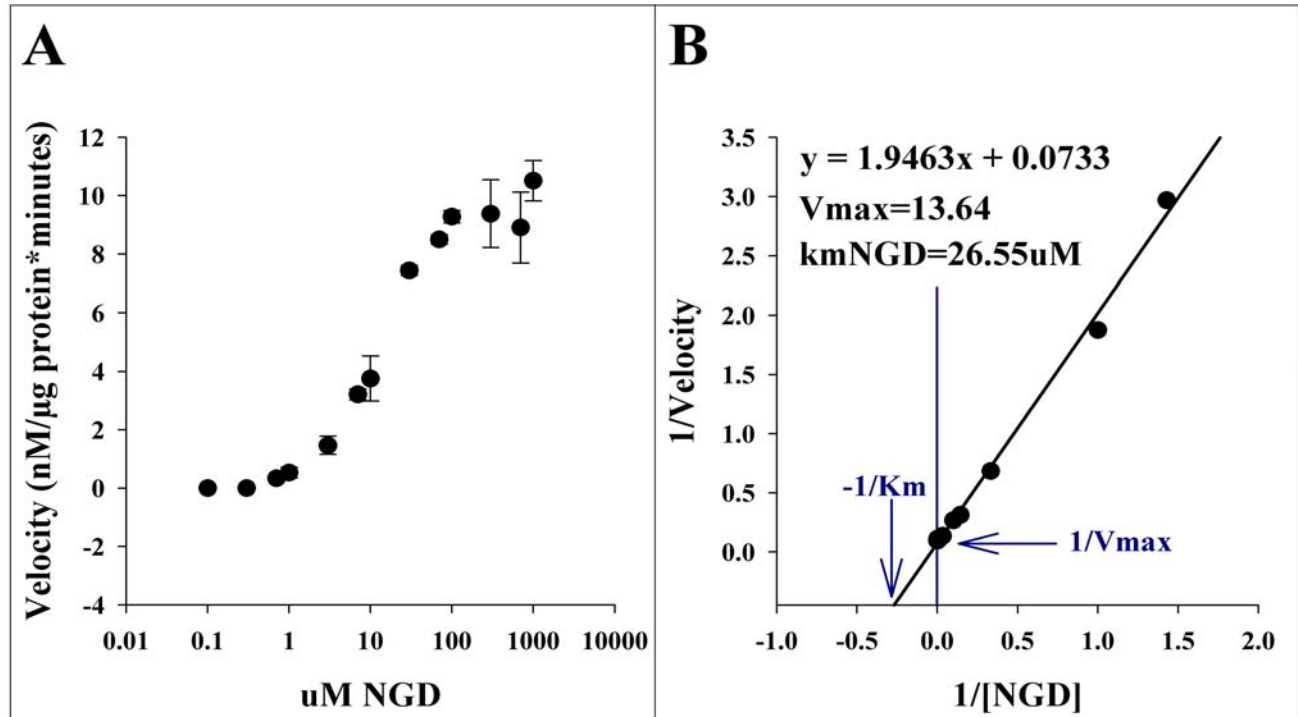
**Figure 2: Enzyme activity of CD38 in the PAG of mice**



Typical Fluorescence chromatograms depicting (A) cGDPR HPLC Standard used for determination of tissue levels of cGDPR and (B) CD38 ADP-ribosyl cyclase enzyme activity leading to the synthesis of cGDPR from the addition of  $\beta$ -NGD<sup>+</sup> to a 40  $\mu$ g sample of PAG. (C) PAG concentration dependent response resulting from the addition of  $\beta$ -NGD<sup>+</sup> to increasing protein concentrations of PAG tissue.

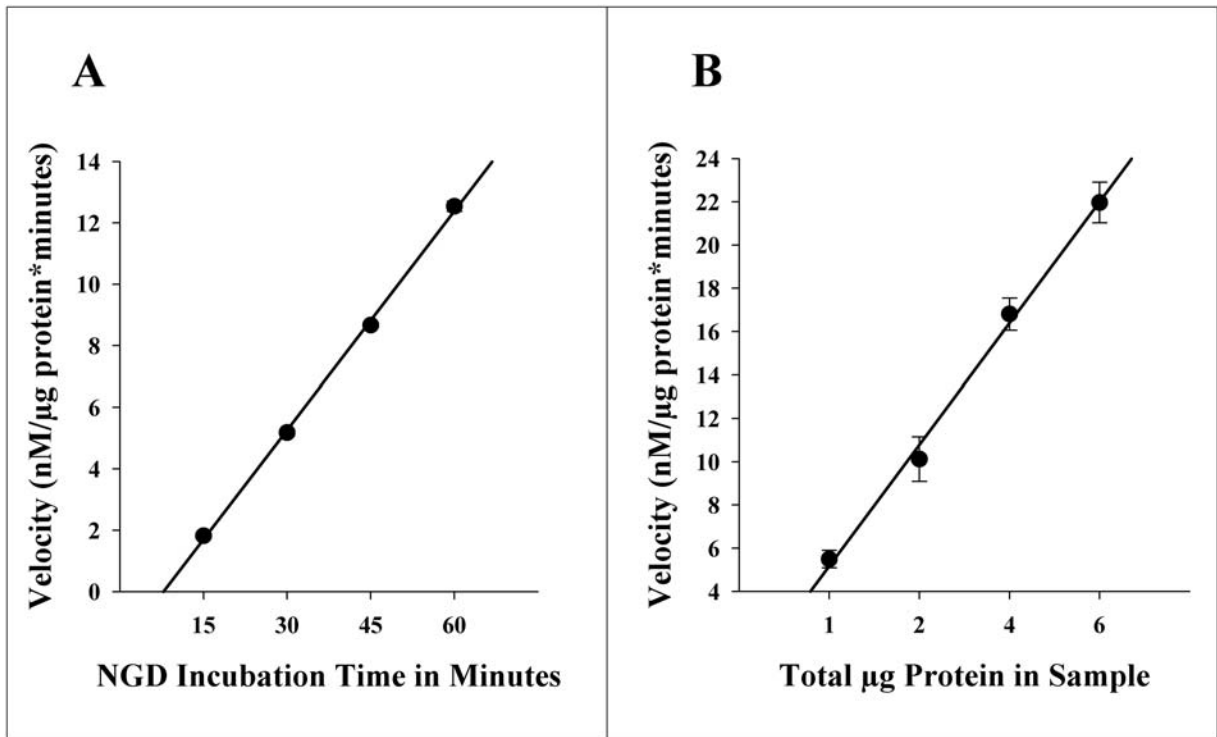
Next we determined the  $K_m$  of CD38 using HPLC analysis of the cyclization of  $\beta$ -NGD<sup>+</sup> to the fluorescent cGDPR in naïve PAG tissue. Figure 3A shows the doses of  $\beta$ -NGD<sup>+</sup> added to the homogenized PAG. In Figure 3B the Lineweaver Burke plot is shown. The  $1/V_{max}$  value was determined from the equation of the line, with the x-intercept set to zero. This value was then used to calculate the  $V_{max}$  and  $K_m$  of NGD<sup>+</sup> for CD38 with the  $K_m$  as 26.55uM. In Figure 4 the effects of varying the incubations time (Figure 4A) and the amount of total protein loaded in the sample (Figure 4B) are shown.

**Figure 3: Enzyme activity of CD38 in the PAG of mice with increasing substrate concentrations**



**(A)** Velocity of CD38 determined at varying concentrations of substrate NGD<sup>+</sup>, n=3. **(B)** Construction of the Lineweaver Burke Plot was used to determine the K<sub>m</sub> of CD38 ADP-ribosyl cyclase.

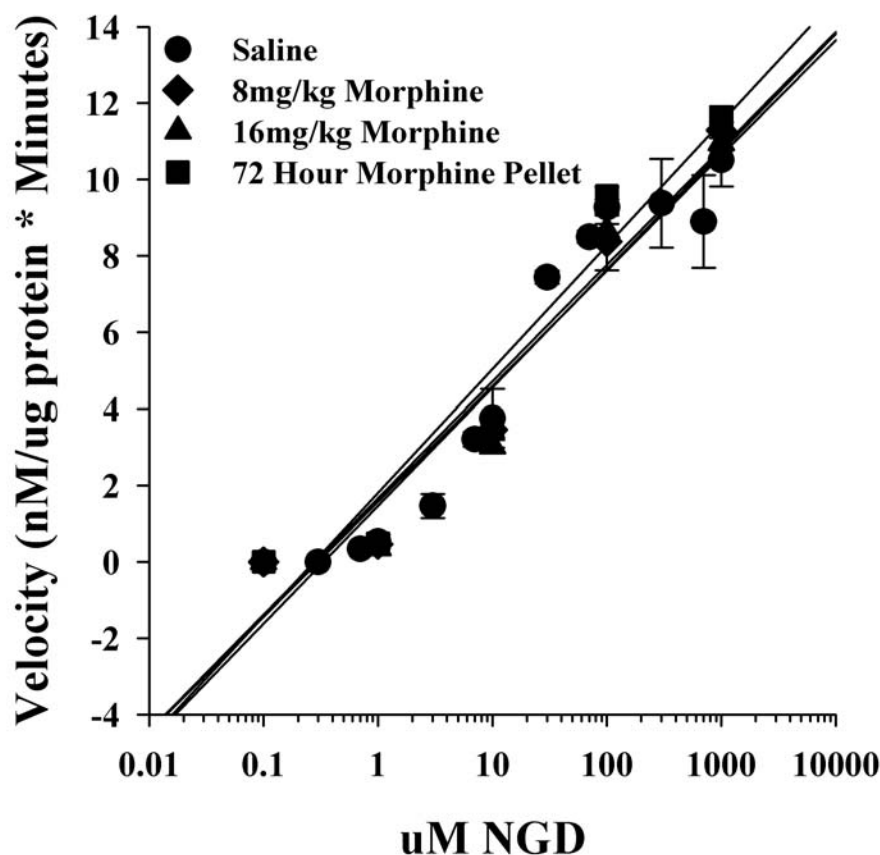
**Figure 4: Enzyme activity of CD38 in the PAG of mice with increasing incubation time with substrate and with increasing total PAG protein in samples**



**(A)** Velocity of CD38 determined at varying incubation times of the sample with substrate NGD<sup>+</sup> at 37°C. Each sample contained 4 $\mu$ g PAG protein, n=5. **(B)** Velocity of CD38 determined with varying  $\mu$ g of PAG protein in the samples. The samples were incubated with 10  $\mu$ M NGD for 30 minutes at 37°C, n=5.

Mice were also administered various acute doses of morphine *in vivo* (8mg/kg or 16mg/kg morphine) as well as allowed to develop tolerance to morphine before sacrifice and HPLC analysis of the conversion of  $\beta$ -NGD<sup>+</sup> to the fluorescent cGDPR by CD38 was measured. We were unable to detect any differences in CD38 activity following any morphine treatment (Figure 5).

**Figure 5: Enzyme activity of CD38 in the PAG of mice after different *in vivo* morphine treatments with varying NGD concentrations**



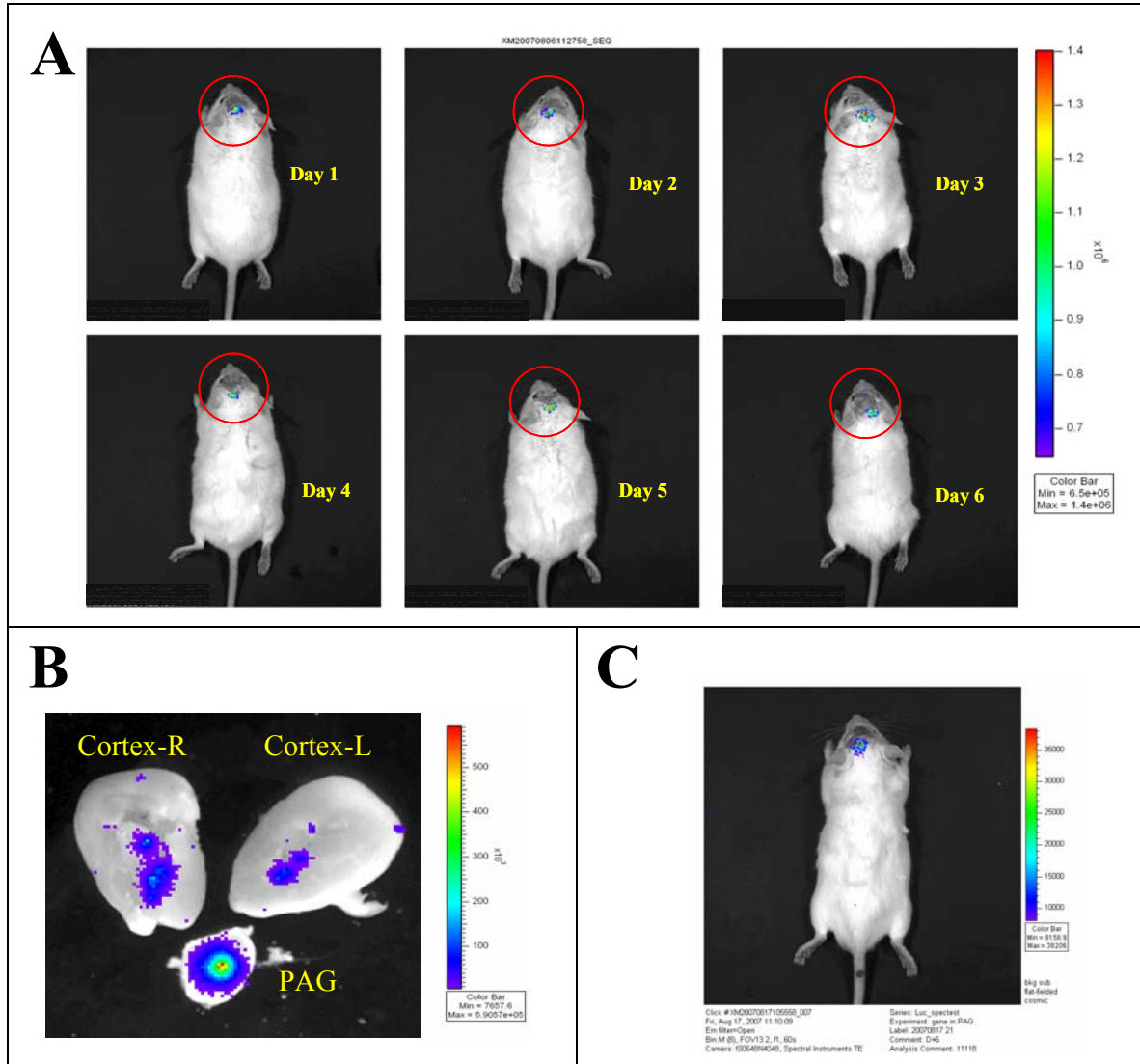
Velocity of CD38 determined at varying concentrations of substrate NGD<sup>+</sup> after different morphine exposures *in vivo*, n=3. Animals were given saline, 8mg/kg morphine, 16mg/kg morphine, or pelleted for 72 hours with 75mg morphine pellets. 30minutes after the acute treatments or at the end of the 72hrs for the chronic treatments animals were sacrificed and the PAG was collected for HPLC analysis. ● indicates saline treated mice, ♦ indicates mice treated with 8mg/kg morphine, ▲ indicates mice treated with 16mg/kg morphine, and ■ indicates the morphine pelleted mice.

To verify that the icv injection was localized to the PAG dynamic monitoring of *in vivo* injection placement was done using the IVIS® 200 Imaging System. The IVIS® 200 Imaging System is an optical system which uses bioluminescent or fluorescent proteins as a reporter which is co-transfected with the target gene. It can visualize gene expression in small living animals for real-time measurements of gene expression. Successful co-transfection of luciferase generates bioluminescence when its substrate luciferin is injected into the animals.

In Figure 6, Panel A, a time course of 6 days is shown indicating that the transfection after icv injection of the luciferase plasmid takes place. Panel B shows the dissected *ex vivo* images of the brain tissue from the same animal in Panel A with the PAG and right and left cortex imaged. This confirmed that the signal seen on the animal skull *in vivo* was derived from the PAG area after the icv injection. In Panel C, the depth of the bioluminescent signal from the surface is quantitated, which is related to the luciferase emission spectrum and the tissue optical properties. The calculated value for the depth from the surface of the head of the animal of luciferase reporter detected was about 2.2 mm, which was approximately the depth of the icv injection. This further confirmed that the signal imaged on the skull surface was propagated from the PAG area.



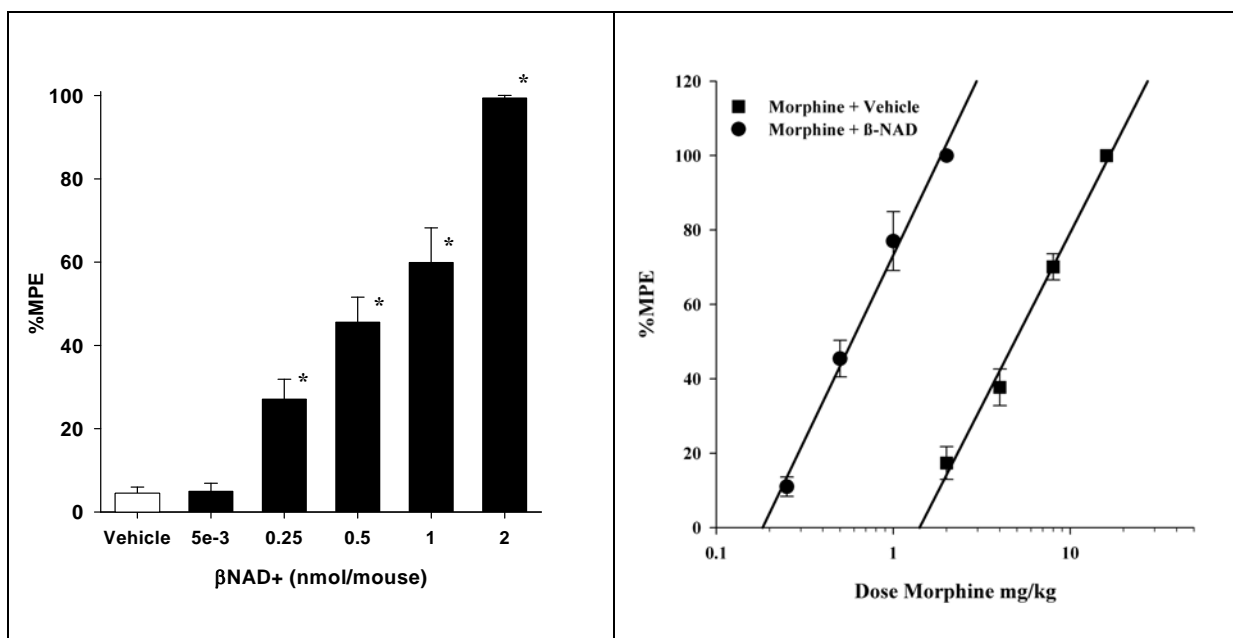
**Figure 6: IVIS imaging verifies the specificity of icv administration**



**(A)** Continuous monitoring of *in vivo* gene expression. During a 6-day continuous observation, the transfected luciferase gene as shown in bioluminescence images (circled in red) can be detected as early as 24 hours after gene transfection. For 6 days, this gene expression was observed every day on the skull surface of animals with Luciferase transfection. **(B)** *ex vivo* image shows the dissected brain from one animal in the group, confirming that the signal on animal skull surface was derived from PAG area transfected with luciferase. **(C)** Spectral Analysis shows the depth of signal in PAG. The depth of the bioluminescent reporters from the surface was quantitated from the image. The calculated value for the depth of luciferase reporter detected was about 2.2 mm, which is around the depth of an icv injection. This further confirms that the developed signal on the skull surface was propagated from the PAG area. Images courtesy of Min Xia.

When added exogenously,  $\beta$ -NAD<sup>+</sup>, a substrate for CD38, can lead to an increase in the production of CD38's products. We administered  $\beta$ -NAD<sup>+</sup> icv in varying doses (Figure 7A) which dose-dependently enhanced the antinociceptive effects of a 2 mg/kg sc dose of morphine, although it had no antinociceptive properties alone. We also constructed morphine dose response curves with  $\beta$ -NAD<sup>+</sup> or vehicle icv administration (Figure 7B) and found that morphine was 7 times more potent in the animals treated with  $\beta$ -NAD<sup>+</sup>.

**Figure 7: CD38 ADP-ribosyl cyclase substrate  $\beta$ NAD<sup>+</sup> increased morphine potency**



**A)** Mice treated with 2 mg/kg morphine sc were injected icv with increasing doses of  $\beta$ NAD<sup>+</sup> and tested in the 56°C tail immersion test. Each treatment group represents 6 mice. **B)** Mice treated with  $\beta$ NAD<sup>+</sup> (2nmol/mouse) and then various doses of morphine sc for construction of dose response curve curves for calculation of ED<sub>50</sub> values and potency ratios. ● indicates animals treated with vehicle and morphine. ■ indicates animals treated with  $\beta$ NAD<sup>+</sup> and morphine. Each data point represents 6 mice.

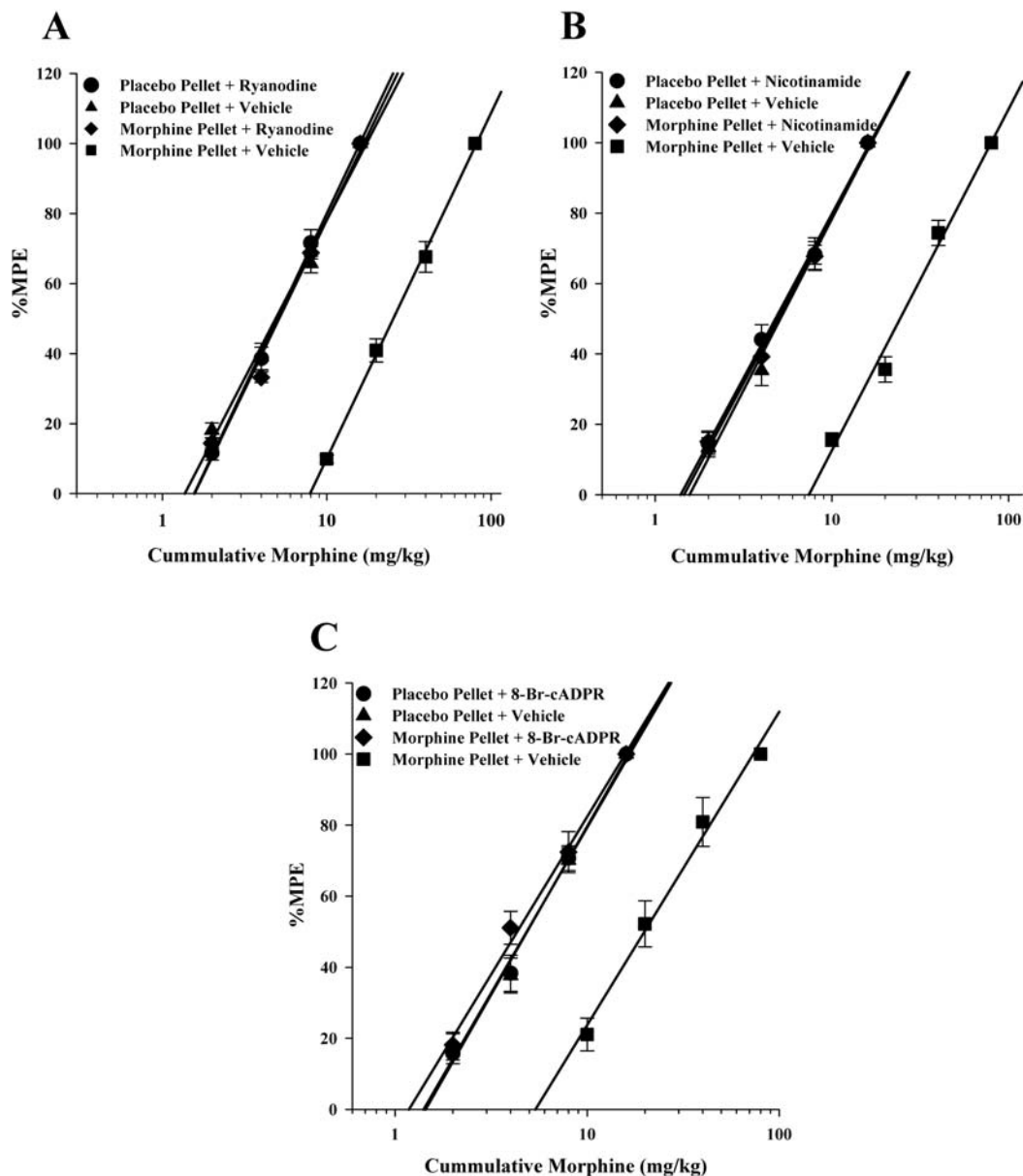
In the next series of experiments, we attempted to elucidate the functional role of CD38 in the mechanism of action of morphine. Nicotinamide is a product of the metabolism of  $\beta$ -NAD<sup>+</sup> to cADPR (i.e. [substrate]  $\beta$ -NAD<sup>+</sup>  $\longrightarrow$  cADPR + nicotinamide [products]) and inhibits CD38 when concentrations are increased to high levels. 8-bromo-cADPR is a chemical analog of cADPR which was developed to be a competitive inhibitor. Ryanodine when used at higher doses can inhibit the ryanodine receptor, the proposed site of action of cADPR. As shown in Table 1 and Figure 8 all three inhibitors were effective at reversing 72 hour morphine tolerance resulting in a 96% reversal with nicotinamide, a 99% reversal with 8-bromo-cADPR and a 96% reversal with ryanodine. These data indicate that the chronic activation of CD38 in morphine tolerance leads to higher activity of the CD38-cADPR-ryanodine receptor pathway leading to disruptions in Ca<sup>2+</sup> homeostasis and higher intracellular free Ca<sup>2+</sup> concentrations.

**Table 1: CD38-cADPR-ryanodine receptor pathway inhibitors**

<b>Treatment</b>	<b>ED<sub>50</sub> value (mg/kg (95% C.L.))</b>	<b>Potency Ratio (95% C.L.)</b>
Morphine Pellet + Ryanodine Morphine Pellet + Vehicle	5.13 (4.87, 5.41) 25.42 (23.80, 27.15)	vs. Morphine Pellet + Ryanodine 4.95 (4.59, 5.33)
Morphine Pellet + Nicotinamide Morphine Pellet + Vehicle	4.94 (4.53, 5.39) 24.28 (22.51, 26.19)	vs. Morphine Pellet + Nicotinamide 4.89 (4.39, 5.44)
Morphine Pellet + 8-Br-cADPR Morphine Pellet + Vehicle	4.32 (3.90, 4.81) 19.86 (17.23, 27.90)	vs. Morphine Pellet + 8-Br-cADPR 4.61 (3.86, 5.49)

Mice were pelleted with 75mg morphine for 72 hours and then given the inhibitor and challenged with morphine. 30 minutes after challenge doses were administered tail immersion latencies were determined for construction of dose-response curves as well as calculation of ED<sub>50</sub> values and potency ratios.

**Figure 8: CD38 inhibitors with morphine 3 day tolerance. Drugs that block the CD38-cADPR-ryanodine receptor pathway reversed morphine tolerance**

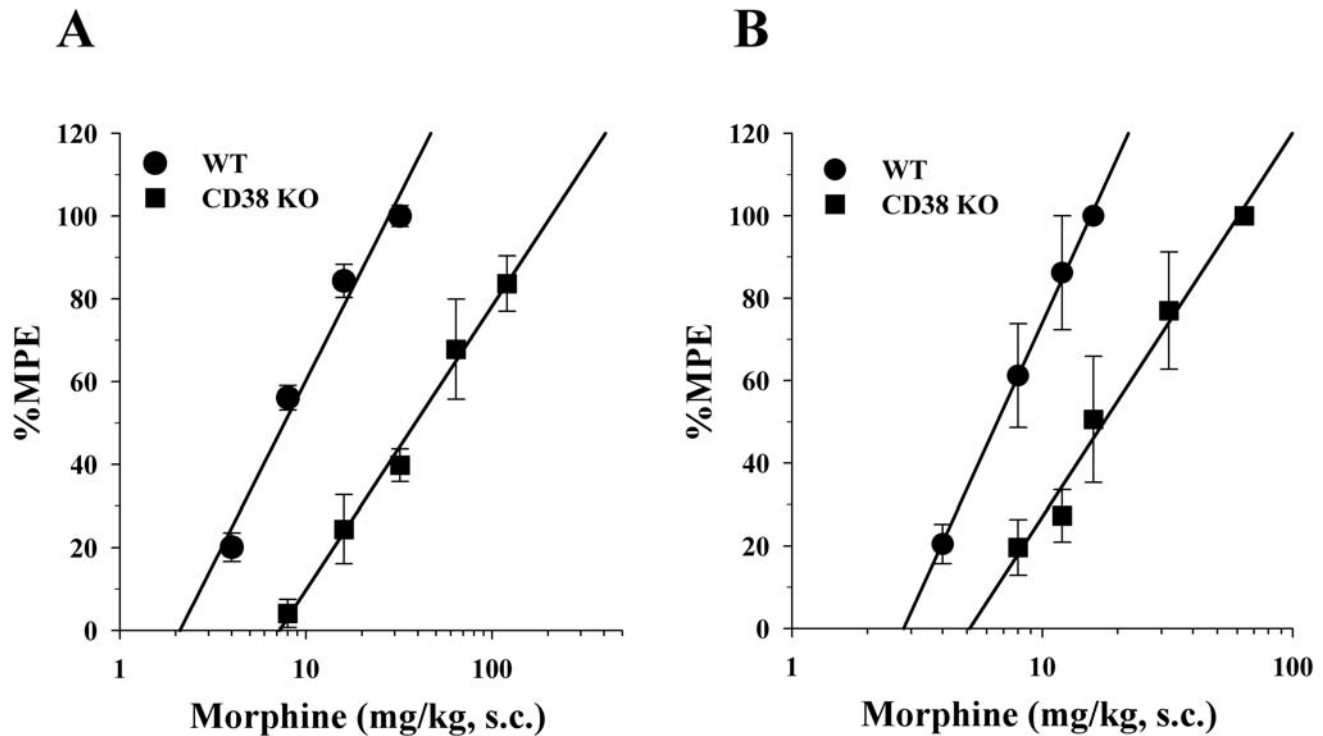


Mice implanted with placebo- or 75 mg morphine-pellets for 72 hours and were then administered inhibitor icv and various morphine doses sc. Antinociception was measured 30 min later and %MPE were calculated and used to construct dose-response curves for calculation of ED<sub>50</sub> values and potency ratios. All 3 inhibitors (A) Ryanodine (1.0 nmol/mouse) (B) Nicotinamide (200 pmol/mouse), (C) 8-bromo-cADPR (10 nmol/mouse) were able to fully reverse 72 hours morphine tolerance. Each data point represents 6 mice. ● indicates placebo pellet with inhibitor, ▲ indicates placebo pellet with vehicle, ◆ indicates morphine pellet with inhibitor, and ■ indicates morphine pellet with vehicle

To further elucidate the role of CD38 in morphine's actions experiments were performed in CD38<sup>-/-</sup> knockout animals. The CD38<sup>-/-</sup> mice were obtained from the colony at the Trudeau Institute, Inc., Algonquin Avenue, Saranac Lake, NY 12983 maintained by Francis Lund. This CD38<sup>-/-</sup> animal was developed as described in Cockayne et al 1998 (Cockayne *et al.*, 1998).

The acute antinociceptive effects of CD38 were assessed using warm water tail immersion and hot plate tests. In the warm water tail immersion we found that morphine had 25% the potency in the CD38<sup>-/-</sup> mice than in the WT animals (Figure 9A). These results were replicated in the hot plate test where morphine was found to be only 17% as potent in the CD38<sup>-/-</sup> mice as in the WT mice (Figure 9B).

**Figure 9: Acute effects of morphine in antinociception assays in CD38<sup>-/-</sup> mice**

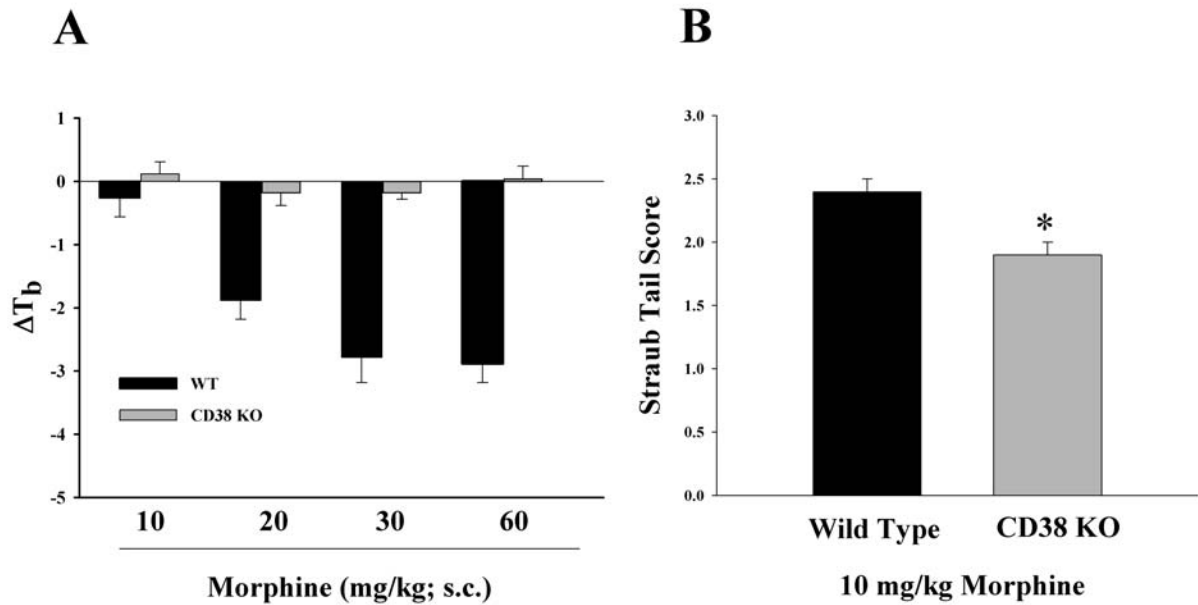


**(A)** Acute effects of morphine in the warm water tail immersion test. Mice were injected with various doses of morphine sc and their tail immersion latencies were determined for construction of dose-response curves 30 minutes later. Data are expressed as mean % MPE  $\pm$  SEM. Each data point represents 6-10 mice. The morphine ED<sub>50</sub> values in mg/kg were as follows: Wild Type = 8.4 (95% C.L. 6.7 to 10.6); CD38 KO = 34.3 (95% C.L. 24.5 to 4.9). Morphine was found to be 4 fold less potent in the CD38 KO mice than in Wild Type mice in this test. **(B)** Acute effects of morphine in the hot plate test. Mice were injected with various doses of morphine sc and their hot plate test latencies were determined for construction of dose-response curves 30 minutes later. Data are expressed as mean % MPE  $\pm$  SEM. Each data point represents 6-10 mice. The morphine ED<sub>50</sub> values in mg/kg were as follows: Wild Type = 6.6 (95% C.L. 5.6 to 7.8); CD38 KO = 39.8 (95% C.L. 18.5 to 85.9). Morphine was found to be 6 fold less potent in the CD38 KO mice than in Wild Type mice in this test.



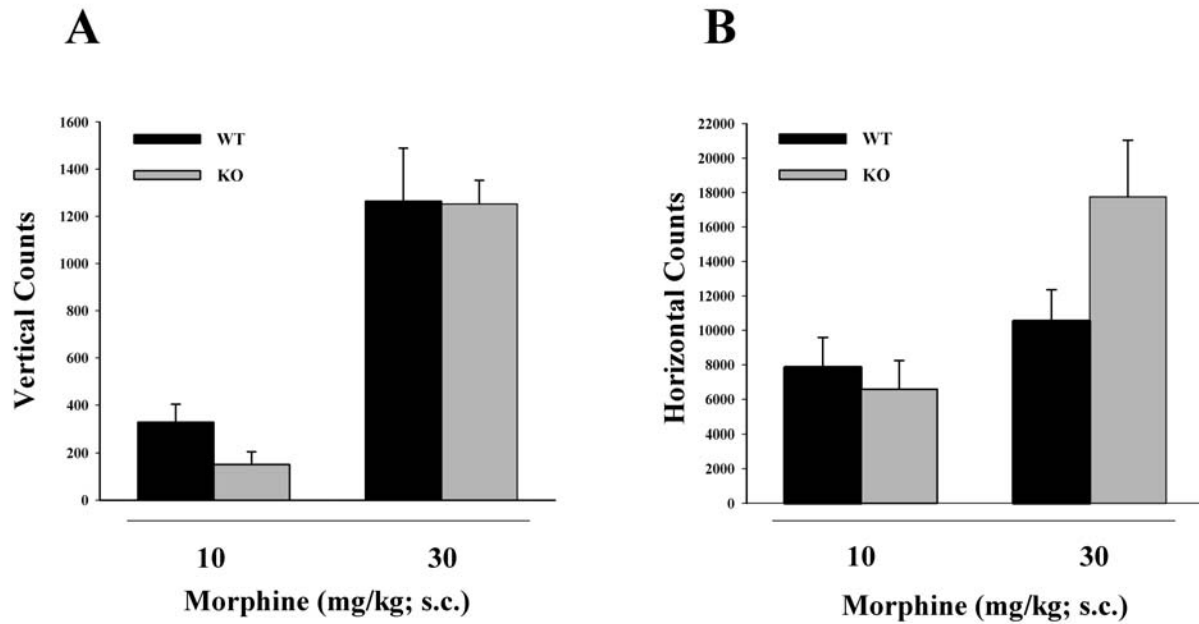
Other effects of acute administration of morphine were also observed in the knockout mice. In morphine induced hypothermia and straub tail measurements, morphine was found to be less potent in the CD38<sup>-/-</sup> mice than in the WT animals (Figure 10). However in spontaneous motor activity (Figure 11) there was no significant difference between the effects of either a 10mg/kg or 30mg/kg dose of morphine in the CD38<sup>-/-</sup> mice and the WT mice in either vertical counts (Figure 11A) or in lateral counts (Figure 11B).

**Figure 10: Morphine induced hypothermia and Straub Tail in CD38<sup>-/-</sup> mice**



**(A)** Acute morphine induced hypothermia. Mice were injected with various doses of morphine sc and test rectal temperatures were obtained 30 minutes later. Results are expressed as the Change in Body Temperature ( $\Delta T_b$ ). In the wild type animals morphine dose dependently induced hypothermia. However, there was no hypothermia found in the CD38 KO animals even at doses where maximal hypothermia was found in wild type animals. Each bar represents 5 mice. **(B)** Acute effects of morphine on straub tail. Mice were injected with 10mg/kg of morphine sc and straub tail was measured 30 minutes later. Each bar represents 6-10 mice. There was a significant reduction in straub tail in the CD38 KO mice compared to wild type ( $p=0.048$ ). Each bar represents 5 mice.

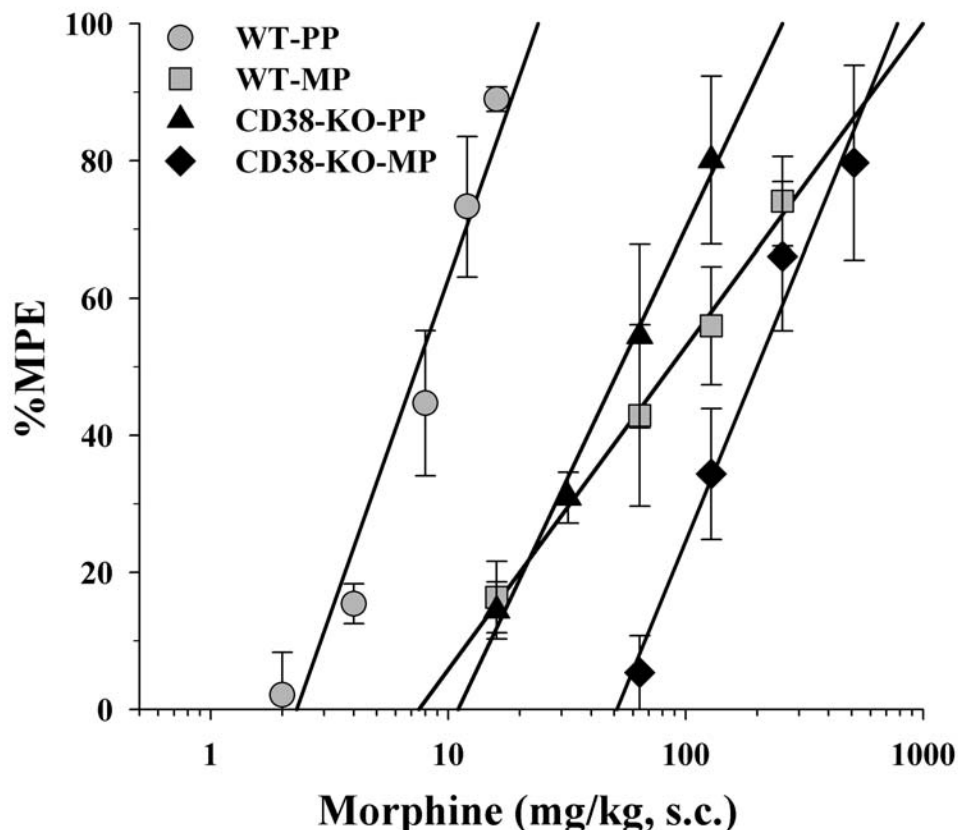
**Figure 11: Acute effects of morphine spontaneous motor activity in CD38<sup>-/-</sup> mice**



Mice were injected with 10mg/kg or 30mg/kg of morphine sc and spontaneous activity was recorded. Each bar represents 5 mice. There was no significant difference between the wild type animals and the CD38 KO animals at either 10mg/kg or 30 mg/kg morphine in either the (A) vertical counts ( $p=0.152$  for 10mg/kg morphine and  $p=0.626$  for 30mg/kg morphine) or the (B) horizontal counts ( $p=0.617$  for 10mg/kg morphine and  $p=0.118$  for 30mg/kg morphine).

We also wanted to determine whether there was any difference between CD38<sup>-/-</sup> and WT animals in morphine tolerance. The data presented in Figure 12 illustrate that tolerance to morphine was developed in both the CD38<sup>-/-</sup> and the WT mice, although there were significant differences between the strains. As was expected from the acute antinociceptive assays, we found morphine to be less potent in the knockout mice both in the placebo treated animals as well as in the morphine tolerant mice. Although tolerance was developed in both CD38<sup>-/-</sup> and WT strains the amount of tolerance developed was different between the strains of mice. In the WT animals 12-fold tolerance developed after the 72hr morphine treatment, but in the CD38<sup>-/-</sup> mice only 3-fold tolerance developed after the same treatment.

**Figure 12: Tolerance to morphine in the warm water tail immersion test in CD38<sup>-/-</sup> mice**



Mice were implanted with either placebo pellet (PP) or 75mg morphine pellets (MP) 72 hrs before they were challenged with morphine. Mice were then injected with various doses of morphine sc and their tail immersion latencies were determined for construction of dose-response curves 30 minutes later. Data are expressed as mean % MPE  $\pm$  SEM. Each data point represents 6-10 mice. The morphine ED<sub>50</sub> values in mg/kg were as follows: Wild Type PP (gray ●) = 7.5 (95% C.L. 6.4 to 8.9); Wild Type MP (gray ■) = 86.8 (95% C.L. 47.9 to 157.4); CD38 KO PP (black ▲) = 54.9 (95% C.L. 40.8 to 73.8); CD38 KO MP (black ◆) = 167.7 (95% C.L. 109.3 to 257.3). Both the wild type mice and the CD38 KO mice developed tolerance to morphine; however morphine was less potent in both the placebo treated mice compared to the wild type and less potent in the tolerant mice compared to wild type. Wild type mice developed approximately 12 fold tolerance in this model, while the CD38 KO mice only developed a 3 fold tolerance.

## V. Discussion

At this time there are no reports of studies that we know of which investigated the role that CD38 may play in morphine antinociception or tolerance. However, CD38 is a logical enzyme to investigate because of its actions on the ryanodine receptor and intracellular  $\text{Ca}^{2+}$  levels - both of which have been implicated in morphine's actions. Therefore, once we confirmed through qualitative RTPCR, western blotting and HPLC assays that CD38 is expressed, present and active in the PAG tissue, we decided to further investigate what effects altering the CD38-cADPR-ryanodine pathway may have on morphine's acute actions and tolerance.

We began our investigation with the icv administration of CD38's substrate  $\beta\text{-NAD}^+$ . This resulted in an increase in the potency of morphine. Exogenous application of  $\beta\text{-NAD}^+$  allows for increased generation of cADPR which leads to increased  $\text{Ca}^{2+}$  release from the ER above and beyond what normally would be released. Increased levels of intracellular  $\text{Ca}^{2+}$  have been linked to tolerance, and the exogenous application of  $\text{Ca}^{2+}$  has been shown to block antinociception (Smith *et al.*, 1995). However, there are also reports of transient increases in intracellular  $\text{Ca}^{2+}$  after opioid administration unrelated to tolerance (Connor *et al.*, 1996; Tomura *et al.*, 1992; Yeo *et al.*, 2001). We did not observe any effect on antinociception after  $\beta\text{-NAD}^+$  application without morphine, therefore we believe that in addition to increased levels of substrate it is necessary for morphine to activate CD38 to cause the increase in cADPR and resultant transient increase in intracellular  $\text{Ca}^{2+}$  which increased the potency of morphine.

Our next goal was to investigate the effect of inhibition of the CD38-cADPR-ryanodine receptor pathway on morphine antinociception and tolerance. We used three separate inhibitors of the CD38-cADPR-ryanodine pathway *in vivo* to determine their effects on acute morphine antinociception and on morphine tolerance. Nicotinamide is a by-product of CD38's enzymatic actions and when added exogenously inhibits CD38 (Berthelie *et al.*, 1998; Inageda *et al.*, 1995). 8-bromo-cADPR is a chemical analog of cADPR and competes with cADPR to inhibit the pathway (Sethi *et al.*, 1997). Ryanodine when used in high concentrations selectively inhibits the ryanodine receptor (Smith *et al.*, 1999).

None of the inhibitors, administered icv, had any effect on the acute antinociception of morphine. Because the three inhibitors all work in different ways to inhibit this pathway it could seem that CD38 is not a part of morphine antinociception. However, both 8-bromo-cADPR and ryanodine specifically inhibit the actions of cADPR (one of three products made by CD38), not CD38 itself. Neither 8-bromo-cADPR nor ryanodine are found naturally in mammalian cells. Ryanodine is an alkaloid found in plants, and 8-bromo-cADPR was specifically fabricated as a competitive analog of cADPR. Nicotinamide on the other hand is the only one of these three compounds which affects the enzyme CD38 in its inhibition of this pathway. Additionally, it is an endogenous compound which is found throughout the body. In the literature nicotinamide is used in *in vitro* experimentation to inhibit CD38. It is possible that our *in vivo* use of nicotinamide had some limitations. One possibility is that it may be quickly broken down by *in vivo* mechanisms not available *in vitro*. It is a by-product of the formation of cADPR, and it would make sense that, if its presence inhibits CD38, there may be a system which

quickly removes it if negative feedback inhibition by this compound is not needed. Therefore, nicotinamide, although injected to the site of interest, may not have been available to inhibit CD38. Another possibility is that nicotinamide may be inhibiting CD38, but only in the production of cADPR. CD38 also produces two other  $\text{Ca}^{2+}$  mobilizing compounds, ADPR and NAADP. It is possible that nicotinamide inhibition of CD38 is in fact an inhibition of the production of cADPR and this shifts the kinetics of the enzyme to produce more of the other two products. It is possible that morphine's acute effects which are dependent on increasing intracellular  $\text{Ca}^{2+}$  levels may be dependent on one of the other two products of CD38, while the tolerance to morphine and the resultant higher basal level of  $\text{Ca}^{2+}$  in the cytosol may be dependent on cADPR. It is already known that multiple  $\text{Ca}^{2+}$  pools are involved in morphine's actions, so it would not be unreasonable to assume that there may be more complexity in their utilization. This hypothesis agrees with the results with *in vivo* administration of  $\beta\text{-NAD}^+$  increasing the potency of morphine, because  $\beta\text{-NAD}^+$  is the substrate for all of CD38's products.

Conversely we found that icv administration of the three inhibitors of the CD38-cADPR-ryanodine pathway all fully reversed 72 hour morphine tolerance. CD38's product, cADPR, leads to an increase in intracellular  $\text{Ca}^{2+}$  levels in the cell, and opioid tolerance is known to correlate with increased basal levels of  $\text{Ca}^{2+}$  in the cell. If CD38 is responsible for these higher levels of  $\text{Ca}^{2+}$ , then inhibiting the CD38-cADPR-ryanodine receptor pathway, and therefore reducing the levels of intracellular  $\text{Ca}^{2+}$ , could be the cause of this tolerance reversal.



Although we have found CD38 to be active in the PAG our results from the HPLC assay were not able to tell us about the effect that morphine has on CD38 activity. From the results of the Lineweaver-Burke plot (double reciprocal plot) the  $K_m$  was determined, using the Michaelis-Menten equation,  $V = V_{max} [S] / K_m + [S]$ , to be 26.55  $\mu$ M. The Lineweaver-Burke plot of CD38's velocity was linear which shows that it is not an allosteric enzyme; this was confirmed by plotting the Eadie-Hofstee Diagram as well (plot not shown).

We were unable to find any difference between the *in vivo* treatments with morphine (acutely 8mg/kg and 16mg/kg morphine and chronically with 72hr morphine pelleting) in the HPLC assay. One possible explanation for this could be the separation in time from the *in vivo* administration of opioid and any resultant changes in CD38 to the HPLC analysis, despite taking measures to keep these events as temporally close together as possible. Even with these precautions and the use of protease inhibitors it is possible that CD38 could have been degraded to some degree between obtaining the sample and the activity assay.

It is known that experimentally CD38 can be found in monomer, dimer and higher oligomer forms, and that the activity increases as CD38 joins together (Chidambaram *et al.*, 1998). However, in kinetic experiments by Suave, it was found that CD38 must be at least in a dimer formation to function properly (Sauve *et al.*, 1998). Their findings indicate that the enzyme dimer forms a single active site per two subunits, which is in agreement with reports that the CD38 homologue *Aplysia* ADP-ribosyl cyclase

crystallizes in dimer form with a deep tunnel created by facing clefts in the subunit which is the active site (Prasad *et al.*, 1996). However, if CD38 was forming higher oligomers as a result of morphine we were unable to detect any increase in activity as a result.

It is possible that any oligomers could have been reduced to dimers in the time between tissue collection and HPLC analysis thus reversing any increased activity.

It is also a potential confound that we only analyzed the PAG. There are many reasons that indicate that the PAG is an appropriate area to study for opioids' actions including its known role in antinociception and tolerance, its high density of MOR, the availability of drugs administered icv to reach it and its extensive use by other researchers with similar goals. However, no experiments have been done which link CD38 to opioids' actions in the PAG and it is possible that by isolating it and only looking for changes in CD38's activity there we overlooked the area where CD38 activity is changed. Another reason we were unable to find any difference could be attributed to the assay itself. Although the protocol was optimized and used successfully in other experiments for the detection of CD38 activity, the PAG is a small piece of tissue. It is possible that any change was too small to detect, or that if the change was localized to a region of the PAG then the homogenization of the tissue and dilution to reach a constant protein concentration could have disguised any change that was present. Also, using NGD<sup>+</sup> as the substrate in the assay could have been a confound. Although it is beneficial to use NGD<sup>+</sup> in this assay because it is an exogenous substance and so all of its product measured is a direct result of the assay and because its fluorescence leads to ease of detection, it has significantly different kinetics with CD38 than NAD<sup>+</sup> does. The percentage of cGDPR generated when

NGD<sup>+</sup> is the substrate is much higher than that of cADPR when NAD<sup>+</sup> is the substrate (Franco *et al.*, 1998; Graeff *et al.*, 1994; Schuber *et al.*, 2004). This altered kinetics of CD38 could have obscured the results which may explain why no difference in levels of product was found. It is also known that the generations of cADPR versus ADPR are competitive pathways with a common intermediate (Sauve *et al.*, 1998). Any alteration to the environment in which the reaction takes place can alter the concentrations of the final product, such as an increase in the amount of a nucleophile (such as water) available. The experimental conditions we used may have unnaturally caused a shift in the concentration of products which may have masked any change in CD38's activity due to the opioid treatments *in vivo*.

In experiments with CD38<sup>-/-</sup> animals it was found that there is a functional role for the CD38 cascade in some of morphine's actions (antinociception, hypothermia and straub tail) but not others (spontaneous activity). This may be partially explained by CD38 playing a role in some of the whole animal inhibitory actions of morphine (resulting in antinociception and hypothermia) but not in other whole animal stimulatory actions (result in spontaneous activity). It is believed that increased activity in the dopaminergic neurons in the mesolimbic dopaminergic pathway play an important role in morphine-induced spontaneous activity (Holstein *et al.*, 2006). However, the stimulatory whole animal effects of morphine are also thought to cause straub tail through the activation of descending dopaminergic systems. This suggests that CD38 may play a more important role in the suppressive effects over the stimulatory effects of morphine.

It is important to address some confusing terminology related to opioids' actions at this point. Opioids' overall inhibition of neurons often results in disinhibition of other downstream neurons which have been tonically inhibited up to that point. This disinhibition results in activation. Similarly it is possible that the transient  $\text{Ca}^{2+}$  increase opioids cause may act to further inhibit these downstream processes due to the activation of an already inhibitory neuron by the opioid.

It is possible that the differences found between the  $\text{CD38}^{-/-}$  mice and the WT mice after the acute administration of morphine could be due to other  $\text{Ca}^{2+}$  mobilizing products of CD38 rather than cADPR. This would be in agreement with the administration of the inhibitors having no effect on the acute antinociception of morphine in Swiss Webster mice while the administration of  $\beta\text{-NAD}^+$  causes morphine to be more potent in nociceptive tests.

It may seem counterintuitive to hypothesize that morphine can stimulate CD38 due to morphine's inhibitory actions on cells in the CNS. However there are indications that morphine can cause release of excitatory neurotransmitters through the disinhibition of their release (Spinella *et al.*, 1996). Additionally there is evidence for glutamate, an excitatory neurotransmitter, to play a role in the expression of CD38 in the CNS (Bruzzone *et al.*, 2004). Therefore it could be hypothesized that the activation of CD38 is not in opposition to morphine's overall inhibitory actions in the CNS, but a direct result of this inhibition affecting the release of activating neurotransmitters and thus activating CD38.

The data presented here involving the CD38-cADPR-ryanodine receptor pathway and morphine makes a strong case for the essential role CD38 plays in morphine tolerance, and possibly in the acute actions as well. Further elucidation of this relationship will aid in further understanding of the mechanism of action of morphine in the production of antinociception and tolerance.

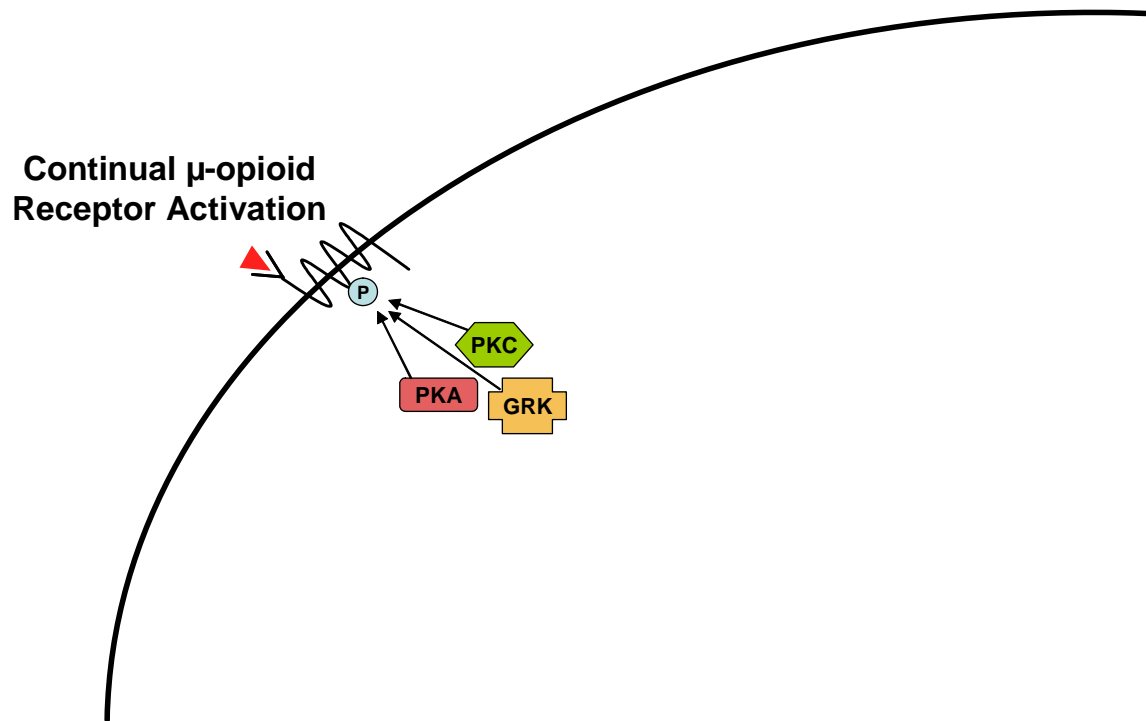
## **CHAPTER 4**

### **General Discussion**

The results of the CD38 experiments taken together with the results of the kinase inhibition experiments help to further our knowledge of the mechanisms which regulate opioid antinociception and tolerance.

It is clear that PKC and PKA phosphorylation of the opioid receptor play an important role in low- and moderate-efficacy opioid tolerance while GRK phosphorylation plays an important role in high-efficacy opioid tolerance (Figure 1). This suggests that desensitization of the MOP is a major mechanism of tolerance for the low- and moderate-efficacy opioids while internalization of the receptor is a major mechanism of tolerance for high-efficacy opioid tolerance. It is also clear that CD38 plays a role in both morphine antinociception and tolerance.

**Figure 1: Phosphorylation of the MOR plays an important role in tolerance**



Now that it is known that these four enzymes play a critical role in opioid tolerance we propose the following hypothesis for their potential interconnected roles in the cell. Altering any one of these enzymes has a major impact on opioid tolerance, therefore it is rational to assume they are each a part of the chain of events that the cell goes through to bring its functions back to normal levels in order to limit the effects of the next exposure to opioids.

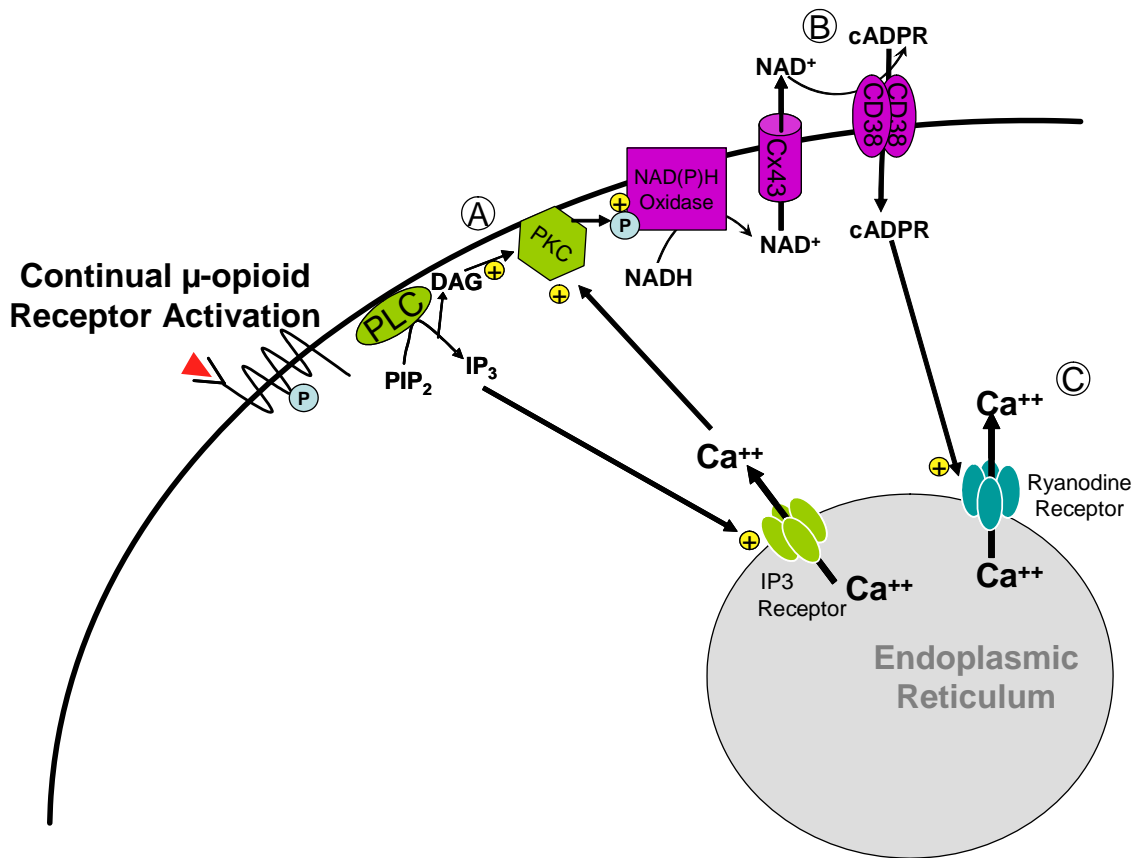
It is known that the levels of activated, membrane bound PKC are increased in opioid tolerance (Mayer *et al.*, 1995). Increased PKC leads to increased phosphorylation on target proteins. One potential target for PKC phosphorylation is NADPH oxidase (Figure 2A). NADPH oxidase is an enzyme complex composed of a catalytic membrane-bound cytochrome b558 heterodimer (gp91<sup>phox</sup> and p22<sup>phox</sup>) that is activated by binding at least three cytosolic components (p47<sup>phox</sup>, p67<sup>phox</sup> and Rac2). Phosphorylation of p47<sup>phox</sup> increases the association of the subunits and activation of the enzyme. Phosphorylation of p47<sup>phox</sup> has been suggested to be caused by PKC (Rotrosen *et al.*, 1990). NADPH oxidase activation results in the conversion of NADH into NAD<sup>+</sup> and generates a reactive oxygen species •O<sub>2</sub><sup>-</sup>.

NAD<sup>+</sup> is then transported into the extracellular space by the transmembrane NAD<sup>+</sup> transporter Cx43. Once in the extracellular space it is available to be converted into cADPR by CD38 (Figure 2B). CD38 both generates cADPR and transports in into the cell. Once in the intracellular space cADPR is able to activate the ryanodine receptor on the endoplasmic reticulum causing Ca<sup>2+</sup> release into the cytosol (Figure 2C). There are



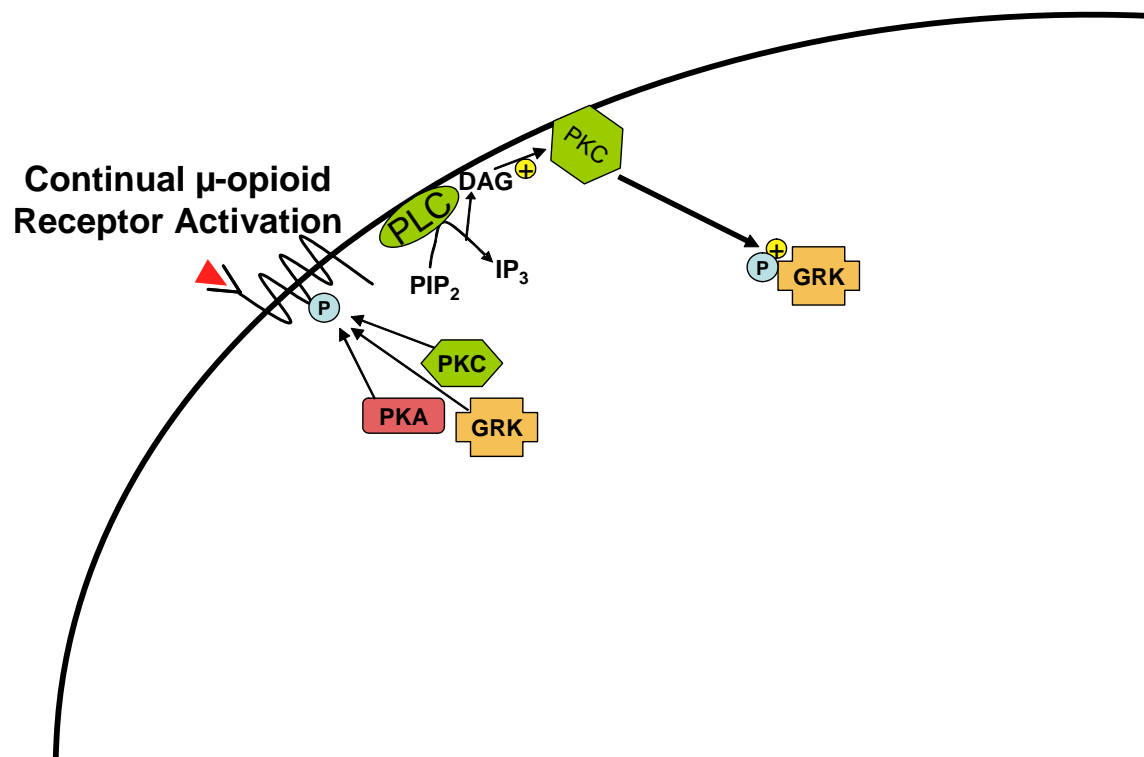
reports in both bovine adrenal chromaffin cells and NG108-15 neuroblastoma x glioma hybrid cells that CD38 is coupled with receptors via G-proteins with experiments using cholera toxin and pertussis toxin implicating Gs and Gi/Go as potential G-proteins involved (Higashida, 1997; Morita *et al.*, 1997). Other work from our collaboration with Dr. Li has found that chronic morphine administration significantly increased the levels of NAD<sup>+</sup> in the PAG, and that the administration of the NADPH oxidase inhibitor diphenylene iodonium blocked this increase. This further indicates a relationship between opioid exposure, NADPH oxidase and the CD38-cADPR-ryanodine receptor pathway.

**Figure 2: Effects of opioid tolerance on the PLC pathway, PKC activation of NAD(P)H Oxidase, activation of CD38 and increased production of cADPR. The cADPR is transported into the cell where it can activate the ryanodine receptor.**



We hypothesize that this is the mechanism responsible for the higher intracellular concentrations of  $\text{Ca}^{2+}$  seen in opioid tolerance. Additionally, this increase in  $\text{Ca}^{2+}$  may further activate PKC. Active PKC phosphorylates the MOR leading to desensitization of the MOR. We believe that MOR occupied by low- and moderate-efficacy opioids possess a conformation which is more readily available to the phosphorylation by PKC and that desensitization through this type of binding plays a primary role in low- and moderate-efficacy opioid tolerance. Additionally, active PKC can also phosphorylate GRK activating it (Figure 3) (Krasel *et al.*, 2001). We believe that when a MOR is occupied by high-efficacy opioids it has an alternate conformation which is more readily available to the phosphorylation by GRKs. The phosphorylation by GRK leads to internalization of the MOR which plays a primary role in high-efficacy opioid tolerance.

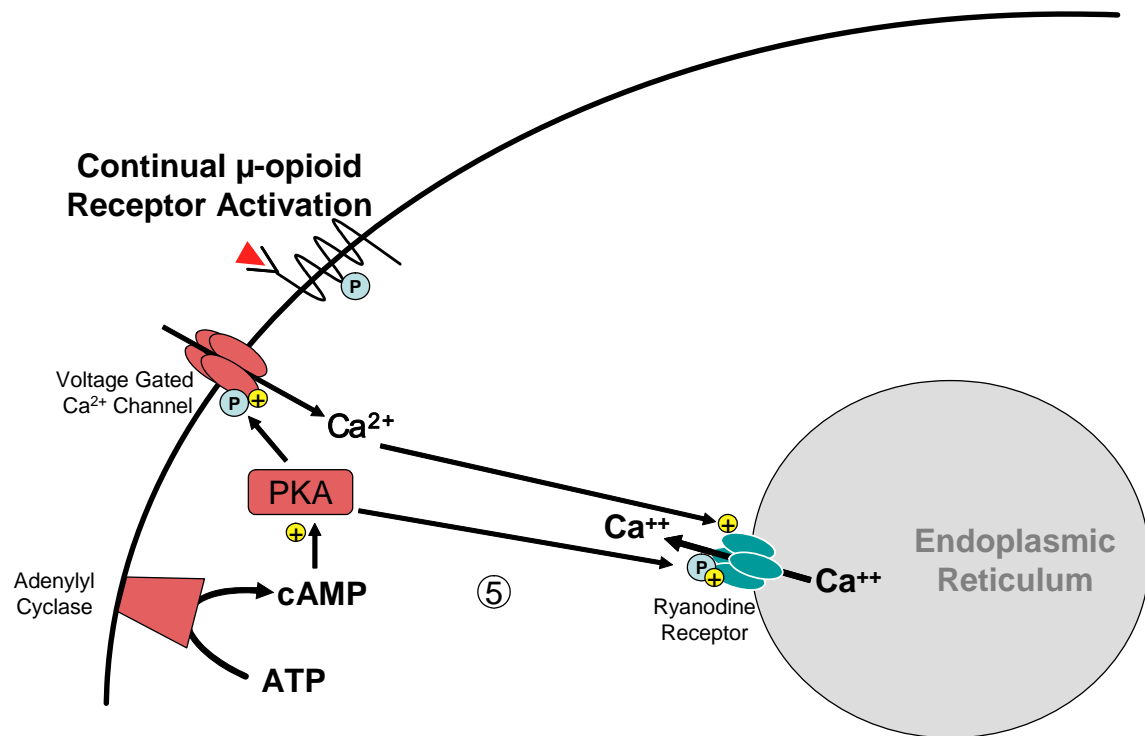
**Figure 3: PKC activates GRK through phosphorylation**



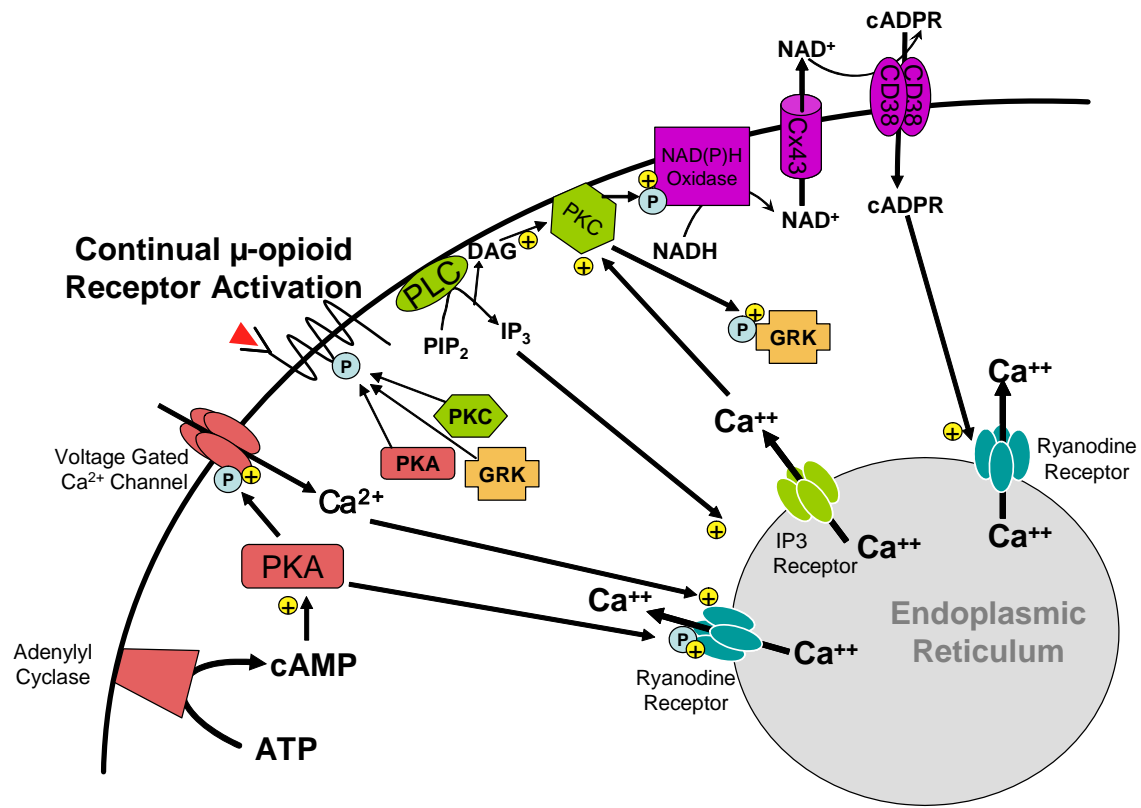
The ryanodine receptor mediated calcium induced calcium release (CICR) is also known to play a role in opioid tolerance (Smith *et al.*, 1999). In CICR  $\text{Ca}^{2+}$  influx into the cell stimulates the ryanodine receptor to further release  $\text{Ca}^{2+}$  from its pools in the ER. This  $\text{Ca}^{2+}$  release can further stimulate other ryanodine receptors on the ER to also release  $\text{Ca}^{2+}$  into the cytosol. There is also evidence in the literature that cADPR is an endogenous modulator of CICR through the interaction with ryanodine and its  $\text{Ca}^{2+}$  pool (Galione *et al.*, 1991; Guo *et al.*, 1997; Meszaros *et al.*, 1993; Sitsapesan *et al.*, 1995).

PKA activity has also been shown to be up regulated in morphine tolerance ((Lane-Ladd *et al.*, 1997; Punch *et al.*, 1997; Self *et al.*, 1995). This kinase has been shown to phosphorylate voltage-gated  $\text{Ca}^{2+}$  influx channels which participate with ryanodine receptors in CICR (Petrovic *et al.*, 2008) (Figure 4). The phosphorylation activated channels allow an influx of  $\text{Ca}^{2+}$  possibly triggering CICR. PKA has also been shown to phosphorylate ryanodine receptors themselves allowing for the release of an inhibitory molecule and allowing  $\text{Ca}^{2+}$  to activate the ryanodine receptor (Marx *et al.*, 2000).

**Figure 4: Activation of the Adenylyl Cyclase pathway in tolerance leads to activation of PKA. PKA also plays a role in CICR**



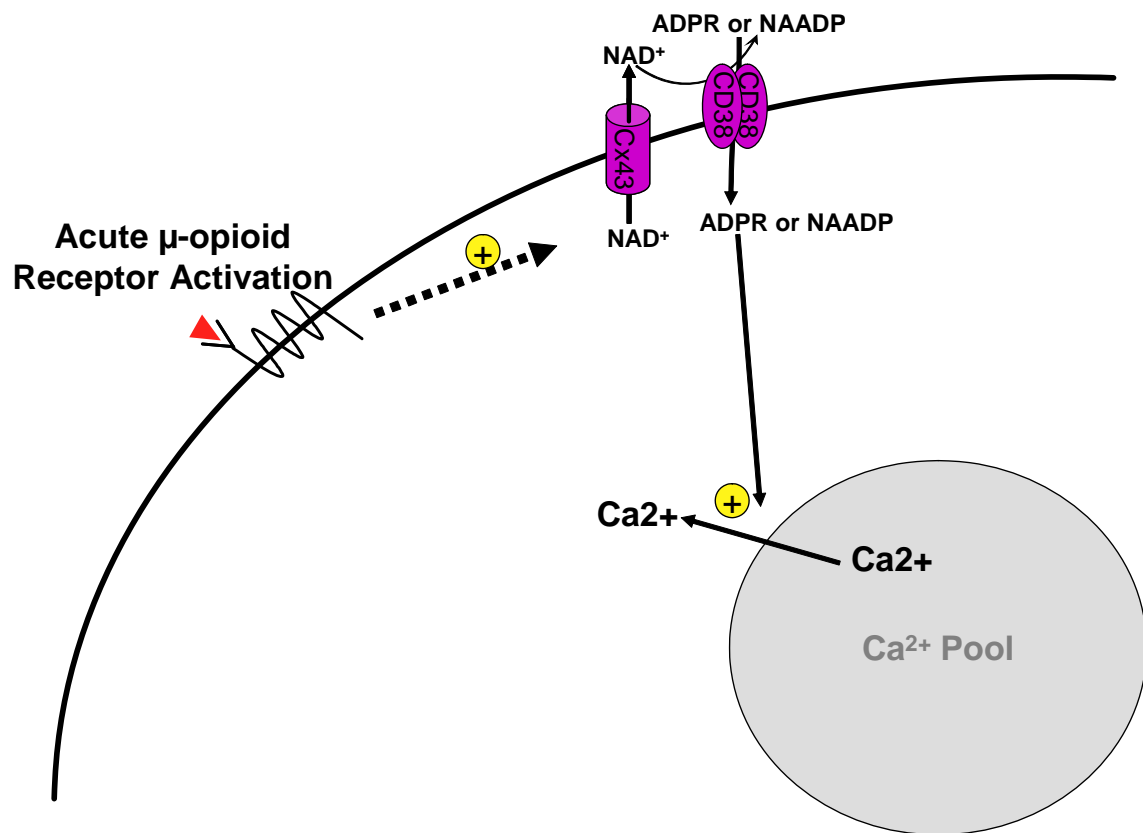
**Figure 5: Overall schematic representation of the mechanisms involved in opioid tolerance**



We also found evidence that CD38 plays a role in the acute effects of morphine. We hypothesize that it is not cADPR but one of the other two CD38  $\text{Ca}^{2+}$  mobilizing products, either ADPR or NAADP, which play a role in the acute effects of morphine (Figure 6). There is evidence that in addition to an overall inhibitory effect on neurons opioids also cause a transient rise in intracellular  $\text{Ca}^{2+}$  levels. We propose that CD38 is the enzyme responsible for this rise in intracellular  $\text{Ca}^{2+}$  after opioid exposure. In our knockout experiments we found that only some effects of morphine were altered in the  $\text{CD38}^{-/-}$  mice. We hypothesize that these effects (antinociception, hypothermia, straub tail) are mediated by the transient  $\text{Ca}^{2+}$  rise and resultant signaling, while the locomotor activation is mediated by the inhibitory effects of opioids. If this is the case then it would be expected that only some, but not all of morphine's acute effects would be altered in  $\text{CD38}^{-/-}$  mice.



**Figure 6: Schematic of acute MOR activation stimulating the formation of ADPR or NAADP**



The results of the experiments in this dissertation further expand our knowledge of opioids' mechanisms of action both in acute administration and tolerance. Particular attention was given to *in vivo* experimentation. It is important to test the findings from *in vitro* and *ex vivo* assays of these pathways in *in vivo* experiments as well. The level of complexity *in vivo* is exponentially greater than that of single cell systems. True understanding of the workings of opioids takes a combined effort of both molecular and behavioral experimentation. It is our hope that the results described herein will further the pursuit of the understanding of opioids' mechanisms of action and tolerance and will aid in the clinical use of opioids to treat pain as well as addiction and tolerance in the future.

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## Vita

Lynn Hull was born on May 5<sup>th</sup>, 1980 in Washington D.C. She grew up in Greenbelt Maryland and attended Eleanor Roosevelt High School. While in high school she participated in school government, swimming (four year letter winner and captain senior year), cross country running, lacross, and played cello in the school's symphony and chamber orchestras as well as in a quartet. Lynn attended Worcester Polytechnic Institute and graduated with a Bachelor's of Science in Biochemistry in 2002. While at WPI Lynn participated in varsity swimming (four year letter winner and captain senior year), varsity rowing (three year letter winner), played cello in the orchestra and in the Medwin Honors String Quartet, was a member of Phi Sigma Sigma and was an Orientation Leader and Peer Advisor for two years. Lynn then worked for two years as a Research Assistant for Dr. Elizabeth Ryder in the Biology and Biotechnology Department at WPI. In 2004 Lynn entered graduate school in the Pharmacology and Toxicology Department at VCU. While a student at VCU Lynn served as Secretary of the Pharmacology and Toxicology Student Organization from 2005-2006, was a founding member of Women In Science and served as the first President of that organization from 2006-2007 and as the Vice President of Fundraising from 2007-2008. Lynn presented twelve posters at national research meetings during her graduate career and gave two oral presentations, winning second place for her oral presentation at the Virginia Academy of Sciences Medical Science Division in 2008.

### Poster Presentations:

*DIG-1, a giant member of the immunoglobulin superfamily, is involved in adhesion in of neuronal processes during development in C.elegans.* L. C. Hull, C. T. Burket, C. E. Higgins, E. F. Ryder – Society for Neuroscience 2003

*Enhanced Expression and Activity of NAD(P)H Oxidase in Mouse Periaqueductal Gray Neurons during Morphine Antinociceptive Tolerance* L. Chen, L. C. Hull, T. T. Lee, F. L. Smith, W.L. Dewey, P. Li – Experimental Biology (ASPET) 2006

*CD38: Novel Interaction with Morphine Analgesic Pathways in Mice* L. C. Hull, T. T. Lee, L. Chen, G. Zhang, P. Li, W. L. Dewey, F. L. Smith – Experimental Biology (ASPET) 2006

*The Role of NAD(P)H Oxidase and CD-38 in the Actions of Opioids* W. L. Dewey, F. L. Smith, P. Li, L. C. Hull, L. Chen – International Narcotics Research Conference (INRC) 2006

*Role of CD38 in Morphine Tolerance* L. C. Hull, P. Li, F. L. Smith, W. L. Dewey – Experimental Biology (ASPET) 2007

*Dynamic In vivo Imaging of NADPH Oxidase Gene Expression to Monitor its Involvement in Morphine's Actions.* Min Xia, L. C. Hull, C. Thomas, B. H. Gabra, N. Li, P. Li, W. L. Dewey – Experimental Biology (ASPET) 2008

*PKC and PKA inhibitors reverse acute tolerance to low- and moderate- but not high-efficacy  $\mu$ -opioid agonists.* L. C. Hull, B. H. Gabra, F. L. Smith and W. L. Dewey – Experimental Biology (ASPET) 2008



*PKC and PKA Inhibitors Reverse Acute Tolerance to Low- and Moderate- But Not High-Efficacy mu-Opioid Agonists.* L. C. Hull, B. H. Gabra, F. L. Smith, and W. L. Dewey – Experimental Biology 2008

*Evidence for an important role of CD38 in acute and chronic effects of morphine.* L. C. Hull, C. Rabender, F. L. Smith, P. Li, B. H. Gabra and W. L. Dewey – Society for Neuroscience 2008

*Alcohol use patterns in patients with chronic pancreatitis and its impact on clinical sequelae (Diabetes, Pain, Exocrine failure).* B. Sandhu, H. Kang, L. Hull, D. Svikis, S. Smith, A. Sanyal – Research Society on Alcoholism (RSA) presented June 2008

*Alcohol use and Chronic Pancreatitis Sequelae in Patients with and without a Paternal History of Alcoholism* L. C. Hull, B. Sandhu, D. Svikis, H. Kang, S. Smith, A. Sanyal – Research Society on Alcoholism (RSA) presented June 2009

*Relationship between Lifetime Drinking Patterns and Medical Complications in a Sample of Patients with Chronic Pancreatitis (CP)* B. Sandhu, L. C. Hull, D. Svikis, H. Kang, S. Smith, A. Sanyal – Research Society on Alcoholism (RSA) presented June 2009

### **Oral Presentations:**

*A Correlation Between Morphine Induced Antinociception and Increased CD-38 in Mouse Brain* L. C. Hull, T. T. Lee, L. Chen, G. Zhang, W. L. Dewey, F. L. Smith, P. Li – CPDD 2006

*PKC and PKA Inhibitors Reverse Acute Tolerance to Low- and Moderate- But Not High-Efficacy mu-Opioid Agonists* L.C. Hull – Virginia Academy of Science 2008 (Second Place in the Medical Sciences Division Oral Presentation Competition)

### **Papers:**

*The C. elegans gene dig-1 encodes a giant member of the immunoglobulin superfamily that promotes fasciculation of neuronal processes* C. T. Burket, C. E. Higgins, L. C. Hull, P. M. Berninsone, E. F. Ryder; Developmental Biology Dev Biol. 2006 Nov 1;299(1):193-205. Epub 2006 Jul 25.